Investigation of neuron T cell interaction in central nervous system tuberculosis

CHOSHI PHUTI SOPHIA

Student Number - CHSPHU001

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Department of Pathology

Faculty of Health Sciences

University of Cape Town

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Supervisors:

Prof Muazzam Jacobs

Dr. Nai-Jen Hsu

DECLARATION

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ABSTRACT

Tuberculosis of the central nervous system (CNS TB) is the severest form of tuberculosis. It is classified as extra-pulmonary tuberculosis due to dissemination of *Mycobacterium tuberculosis* (Mtb) bacilli from the lung to the brain. It affects mostly children and immune suppressed individuals and high incidents of death occur as a result of missed diagnosis and delayed treatment. Therefore, the is a need for improved therapeutic strategy and a better understanding of the CNS immunity; investigate cells targeted for infection, their respective response to infection and interaction with different cell types to the overall protection of the CNS - as accumulating evidence indicates a dynamic neuronal lymphocyte interplay that defines outcomes of diseases. A novel observation was previously made, that neurons are infected by Mtb during *in vitro* and *in vivo* infection. The aim of this study was to further characterize neural responses induced by mycobacteria using hippocampal primary neuron cultures, infected with H37RV and BCG. Secondly, this study investigated the importance of interaction between neurons and immune cells in immunity against mycobacterial challenge using an optimised neuron T cell co-culture model. Investigation included identifying the production levels of neuronal cell surface markers and cytokines induced by Mtb. In flow cytometry and ELISA analyses, infection exhibited a robust inflammatory response with increased neuronal production of cytokines such as IL1β, IL6, TNF and regulatory cytokine IL10 in vitro and in vivo. Neuronal MHC class I expression was upregulated by infection, suggesting possible antigen dependent interactions between neuron and $CD8^+$ T cells. In co-cultures, neurons induced expression of Tbet, RorγT and Gata3 T cell transcription factors through direct contact with T cells. These data highlighted the likelihood of neurons activating T cells upon mycobacterial stimulations. It may potentially be utilised to broaden the understanding of CNS immunity under pathological conditions and possibly lead to identification of novel immunomodulatory targets that could be exploited for new rapid sensitive diagnostics and early opportune intervention against CNS TB – reducing morbidity and mortality associated with the disease.

LOCAL CONFERENCES AND MEETINGS

- **Phuti Choshi**, Nai-Jen Hsu, Muazzam Jacobs. Investigation of the interaction between neurons and T cells in central nervous system tuberculosis. 2nd African College of Neuropsychopharmacology Congress, July **2016**, Stellenbosch, Western Cape, South Africa *(Oral).*

- **Phuti Choshi**, Modelling central nervous system tuberculosis Invitro. University of Cape Town Neuroimmunology Meeting, December **2015**, Kalk Bay, Western Cape, South Africa *(Oral).*

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BCG-GFP	Bacillus Calmette-Guerin-green fluorescent protein
CNS-TB	Central nervous system tuberculosis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescently activated cell sorting
IL	Interleukin
MHC I	Major histocompatibility complex I
MOI	Multiplicity of infection
Mtb	Mycobacterium tuberculosis
RT	Room temperature
TB	Tuberculosis
TGF	Transforming growth factor
TNF	Tumour necrosis factor alpha
WT	Wild type

1. INTRODUCTION

1.1. The Epidemiology of Tuberculosis

Tuberculosis (TB) remains one of the most life threatening communicable diseases in the world, claiming millions of lives annually. In 2015, 10.4 million people estimated to have fallen ill with TB and 1.4 million cases succumbed to the disease (WHO, 2016). Globally, 11% of new TB cases were co-infected with HIV. TB now outranks HIV as the infectious leading cause of death worldwide (WHO, 2016). This is despite the disease being largely curable and the progressive improvements in access to TB treatment in the last decade.

1.2. The Pathogenesis of Tuberculosis

Tuberculosis is caused by an intracellular bacterium, *Mycobacterium tuberculosis* (Mtb), and is transmitted when the organism is aerosolised by the cough of an infected patient and inhaled into the alveoli of a new host (Alland et al., 1994). The introduction of Mtb bacilli into the lungs can cause respiratory infection characterised pathologically by necrotic granulomas, termed pulmonary TB which can later disseminate to other organs resulting in extra-pulmonary TB (Rieder et al., 1990, Solovic et al., 2013). In the lung, after aerosol inhalation, bacteria are taken up by resident macrophages and a series of complex interactions with the host take place, more macrophages are recruited to the site, specific T cells begin to accumulate, and a granuloma is formed (Ramakrishnan, 2012). The statistics show that one-third of the world's population is infected with Mtb but only 12% of these individuals actually develop the disease (Vynnycky and Fine, 1997) and a successful arrest of the infection results asymptomatic and non-transmissible latent infection (Smith, 2003). Before the infection is contained in granulomas, bacilli are filtered into draining lymph nodes, where there is a low-level bacteraemia in which Mtb disseminates to distant sites in

the body resulting in extra-pulmonary TB (Solovic et al., 2013). Of all TB cases, ~ 80% are pulmonary TB with 15% of the burden due to extra-pulmonary TB. Extra-pulmonary TB is a disease outside the lungs, can be found in nearly every organ system including the central nervous system (CNS) (Solovic et al., 2013).

1.3. Central Nervous System Tuberculosis (CNS TB)

Mtb is the most common cause of CNS infection (John et al., 2015) with the most global burden in sub-Saharan Africa and Asia (Berman et al., 1992, Deeny et al., 1985, van Well et al., 2009). Central nervous system tuberculosis (CNS TB) affects 1-5% of all active TB cases of tuberculosis and it is associated with high morbidity and mortality (Thwaites et al., 2013, Garg, 1999). It mostly affects children and immune suppressed individuals (Okike et al., 2014, Vinnard and Macgregor, 2009). It is the severest form of TB caused by haematogenous dissemination of Mtb to the brain followed by the development of Rich foci (small tuberculous lesions) in the brain, spinal cord or meninges and remains dormant. Rupture or growth of the lesions into the subarachnoid space leads to disease development (Isabel and Rogelio, 2014, Donald et al., 2005) (Figure 1-1). CNS tuberculosis may manifest itself as tubercular encephalitis, intracranial tuberculomas, or a tuberculous brain abscess and most commonly as tuberculous meningitis (TBM) (Be et al., 2009). After the release of tubercle bacilli from granulomatous lesions into the subarachnoid space, a dense gelatinous exudate forms - containing variety of cells including lymphocytes. Such immune host factors determine whether the infection is contained and if the dissemination of the bacilli leads to clinical disease, in a case of excessive inflammation (Isabel and Rogelio, 2014). The host immune response contributes to defence against invading pathogens and subsequent damage to the nervous system (Bailey et al., 2006).

Improved understanding of the pathogenesis of CNS TB, type and role of specific cells involved, the realization of the cause of chronic neurological, cognitive and behavioural disease may bring about efficient prevention of infection, limit the persistence of neuronal dysfunction and enhance neuroprotection.

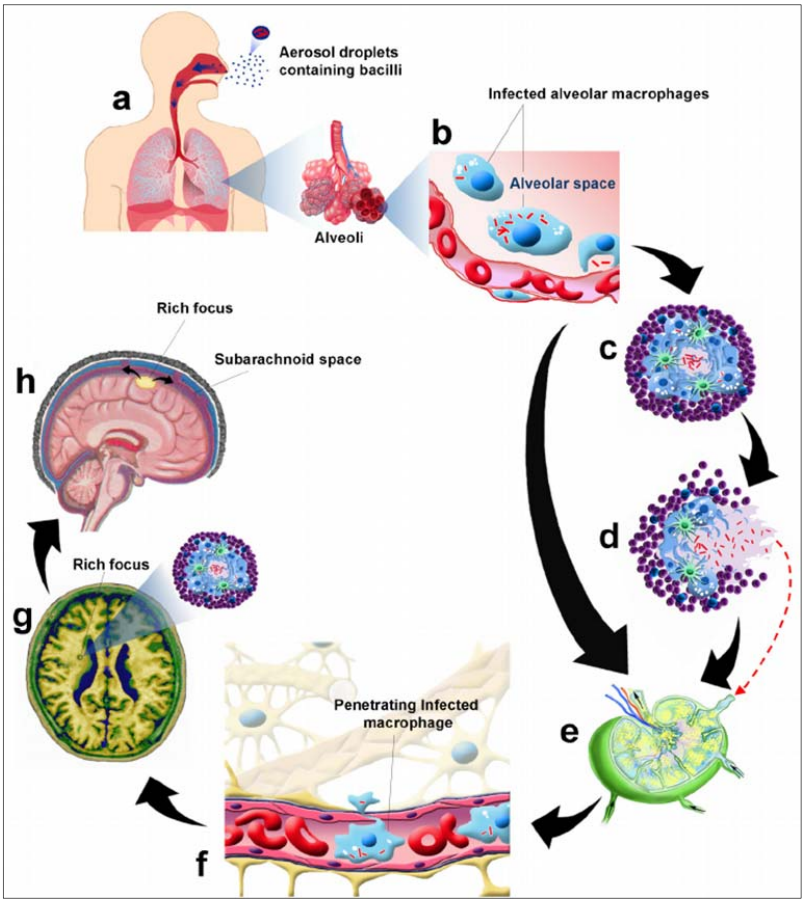


Figure 1-1: Schematic illustration of the pathogenesis of tuberculous meningitis (TBM) postulation of the formation of Rich foci. (a) Aerosol transmission of Mtb (b) Phagocytosis of Mtb by alveolar macrophages inside alveoli. (c) Granuloma formation in the lung (d) Mtb escapes from the granuloma (e) Mtb bacilli are filtered into draining lymph nodes (f) After spreading through the blood circulation, Mtb can enter the CNS through the blood brain barrier (BBB). (g) Bacilli seed to the meninges or the brain parenchyma, forming Rich foci.

1.3.1. Different cell types targeted for infection in the CNS including neurons

Mtb encodes specific proteins that actively facilitate entry into cells in order to establish infection. Several studies have reported on different cell types targeted by Mtb for invasion (Wolf et al., 2007, Teitelbaum et al., 1999, Munoz et al., 2009, Garcia-Perez et al., 2003). Amongst these, macrophages are well described as preferred host cells in the lung (Stanley and Cox, 2013, Pieters, 2008, Cambier et al., 2014). Other cells such as dendritic cells are also infected by Mtb at a higher rate than previously thought (Wolf et al., 2007).

In the CNS, microglial cells are regarded as the resident macrophages and principal target for Mtb (Curto et al., 2004, Rock et al., 2005, Rock et al., 2004b). This was demonstrated in a culture of mixed glia where Mtb showed selective association with microglia than astrocytes (Rock et al., 2008, Rock et al., 2005). Nonetheless, neurons, microglia and astrocytes are all potential hosts for pathogen in the CNS (Rock et al., 2004a, Rambukkana et al., 2002). An early study has found cultured rat dorsal root ganglion neurons could be invaded by intracellular bacterium *Listeria monocytogenes* (*L. monocytogenes)* efficiently by internalization and also via cell-to-cell spread from infected phagocytes (Dramsi et al., 1998). Later, neurons were shown to control *L. monocytogenes* infection in an interferon gamma (IFN-γ) dependent manner (Jin et al., 2004, Jin et al., 2001). *Mycobacterium leprae (M. leprae)* is another intracellular bacterium that has been shown to be associated with neurons. The study indicated that *M. leprae* invasion is not the cause of demyelination but the bacterial attachment alone is sufficient to induce nerve pathology via contact dependent mechanisms (Rambukkana et al., 2002). The recent observation of neurons as Mtb hosts is a

novel discovery demonstrated by *in vitro* and *in vivo* infection of neurons by Mtb (Randall et al., 2014).

1.4. Immune response in CNS homeostasis and disease

CNS immunity is comprised of innate and adaptive immunity operated by CNS-resident cells and infiltrating immune cells. Almost all pathological changes within the CNS elicit a prominent inflammatory reaction (Lucas et al., 2006, Klein et al., 2017). CNS inflammation in response to pathogen infection is a highly regulated process to limit potential pathological damage. The nature of inflammation in the CNS is often not well defined due to the notion of the CNS as an immune privileged site. However, there is new understanding that CNS is not as immune privileged as previously described (Louveau et al., 2015, Gadani et al., 2015). T cells can gain entry into the CNS under physiological conditions to constantly survey for maintenance of the CNS homeostasis, for resolution of infections, degeneration and tissue damage (Engelhardt and Ransohoff, 2005, Ransohoff and Engelhardt, 2012). Not only can immune cells cross the blood-brain-barrier (BBB), the resident CNS cells (neurons, microglia) play a huge role in regulating inflammatory immune responses (Sternberg, 2006, Tian et al., 2009, Tracey, 2002). These resident cells express specific adhesion molecules to maintain the CNS homeostasis and to prevent excessive inflammation.

When microglia and astrocytes are infected with mycobacteria, high levels of proinflammatory cytokines are produced (Rock et al., 2005).The release of cytokines such as tumour necrosis factor (TNF), interleukin 1 (IL1), interferons etc. and production of chemokines recruit immune cells to the site of infection and form part of host defence mechanisms in the subarachnoid space, increasing the efficiency to eliminate pathogens. Production of TNF alpha (TNF-α) during CNS inflammation plays a key role in

inflammatory response. Moreover, it has been shown that TNF-α is essential for host protective immunity against CNS TB (Francisco et al., 2015a). High interleukin 4 (IL4) expression was also found in H37Rv infected brain (Isabel and Rogelio, 2014) and molecules such as transforming growth factor beta (TGF-β), interleukin 10 (IL10) and IL4 in neuroinflammation downregulate proinflammatory molecules and dampen immune responses (Hendrix and Nitsch, 2007) in an immune regulatory manner, displaying their neuroprotective activities. CNS injury is associated with a T helper 2 (Th2) skew (Walsh et al., 2015, Gadani et al., 2015) which in the context of CNS mycobacterial infection may be important in down regulating T helper 1 (Th1) responses and facilitating neuronal survival. The role of T cells in CNS viral infections is well established, regulatory IL10 down regulate T helper 17 (Th17) driven inflammation (Kulcsar et al., 2014, Martinez et al., 2012). IFN-γ producing CD8+ T cells are important in restraining CNS viral infection and preventing re-infection (Verjans et al., 2007, Khanna et al., 2003). $CD4^+$ and $CD8^+$ T cells act together in addition to TNF-α and IFN-γ and they use perforin and Fas ligand to potentiate cytotoxicity of viral infected cells (Stohlman et al., 2008, Tishon et al., 2006). In other neuroinflammatory diseases such as Multiple Sclerosis (MS), insufficient regulatory responses by T regulatory cells (Tregs) contributes to CNS pathology due to their inability to reduce inflammatory T cell (IFN-γ producing Th1 and IL17 producing Th17 cells) responses (Liblau et al., 2013, Luchtman et al., 2014). The amount of FoxP3 expression by Tregs is directly proportional to their capacity to suppress inflammatory T cells (Venken et al., 2008, Liu et al., 2015). The balance between Tregs cells and inflammatory T cells is important for CNS homeostasis and preventing neuroinflammation.

1.4.1. Neuron T cell interaction

Several studies have identified various neuronal pathways that regulate immunity and inflammation. For a while the role of neurons in regulation of CNS inflammation was addressed mainly through their interaction with CNS resident antigen presenting cells. Neurons were not viewed as players in control and modulation of immune responses. However, recent research applying immunological methods to neurological studies have highlighted that neurons function as immune regulators through their control on glial cells and infiltrated T cells. Neurons have been shown to induce T cell apoptosis (Flugel et al., 2000) and inhibit immune responses through contact and contact independent mechanisms (Tian et al., 2009). In experimental models of MS, neurons can directly alter the phenotype and function of CD4 T-cells (Liu et al., 2006).

The role of neurons in the immunological defence against CNS pathogens has always believed to be passive because neurons do not express major histocompatibility complex (MHC II). However, neurons can express major histocompatibility complex (MHC I) molecules in the CNS (Shatz, 2009, Foster et al., 2002, Chacon and Boulanger, 2013, Cebrian et al., 2014). The expression has been shown in response to viral infections (Chevalier et al., 2011) and exposure to IFN-γ (Neumann et al., 1995). The expression of MHC I and not MHC II on neurons have suggested that antigen dependent responses may be confined to neuron - $CD8^+$ T cell interaction (Chevalier et al., 2011). Neuronal MHC I can interact with Cis and Ly49 receptors of immune cells (Held and Mariuzza, 2008, Scarpellino et al., 2007) and has also been shown to signal through T cell receptor co-receptor CD3 in the brain (Syken and Shatz, 2003). In CNS viral infections neuron $CD8^+$ T cell interaction induces cytotoxic mediated killing of infected neurons (Meuth et al., 2009, Medana et al., 2000, Chevalier et al., 2011, Cebrian et al., 2014). However, mechanism utilized by T

lymphocytes to control pathogens varies, and $CD8^+$ mediated killing may include Fas ligand, perforin and/or granzymes (Liblau et al., 2013). Neurons can upregulate surface expression of Fas upon infection and this promotes pathogen clearance by inducing death of infected cells (Figure 1-2).

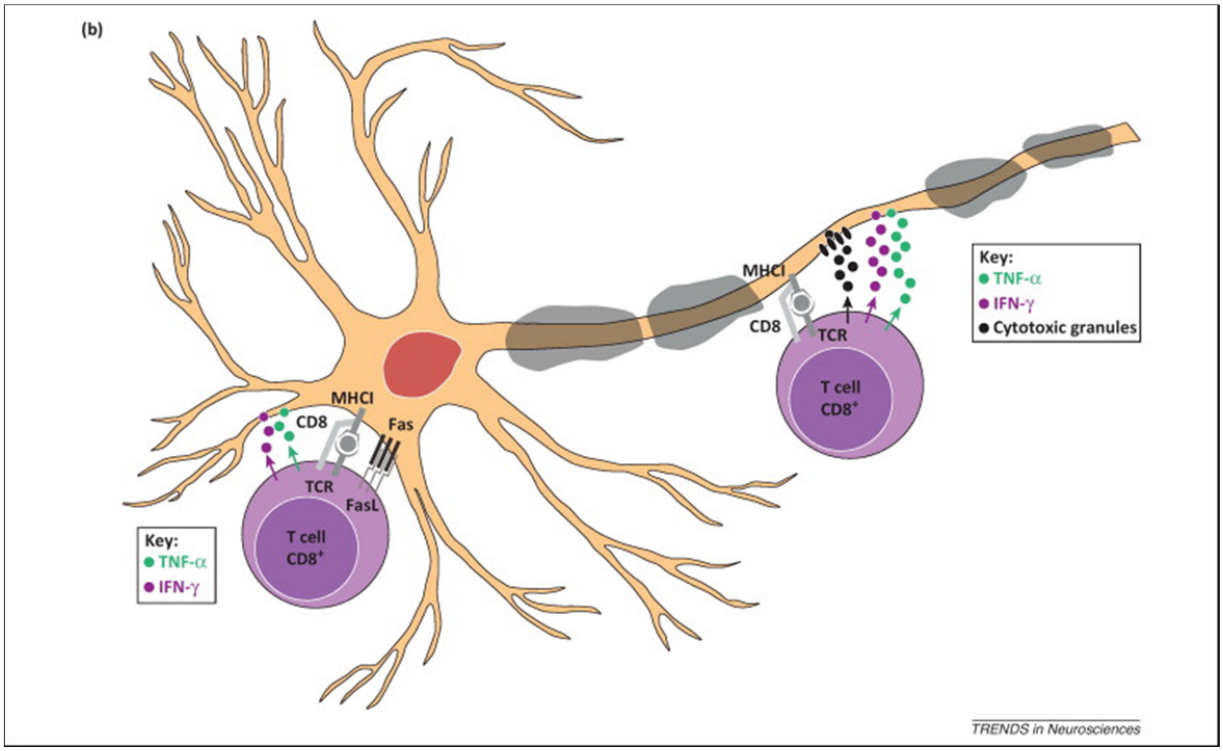


Figure 1-2: Schematic illustration of the consequences of antigen-specific CD8 T cell interactions with neurons. Reproduced from: (Liblau et al., 2013). T cell receptors (TCRs) recognise antigenic peptides associated with MHC class I molecules. Following activation, CD8 T cells cytotoxic factors such as perforin and granzymes A and B contained in secretory lysosomes called lytic granules CD8 T cells also release cytokines such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α which affect the biology of the neurons that express their receptors, sensitizing them to apoptosis (Neumann et al., 1997, Mizuno et al., 2008) . Fas ligand at the cell surface of the CD8 T cells can engage Fas expressed by neurons and initiate an apoptotic cascade (Zohar et al., 2008, Medana et al., 2000, Cebrian et al., 2014).

Neuron T cell interactions are not only confined to CD8+ T cells in CNS inflammation. Three major subsets dominate the T cell inflammation profile, namely; $CD4^+$, $CD8^+$ and

regulatory CD4+ T cells. Each sub-population has distinct functions within the CNS. All T cell subsets are all important under homeostatic physiological conditions and control of inflammation. However, regulatory T cells have been shown to contribute more to CNS homeostasis and most importantly, counter balancing inflammation in a less regenerative CNS setting. T cell responses in the brain have both beneficial and detrimental effects on brain function (Gadani et al., 2015). Surface molecules expressed on T cells reflect their maturity and activation, thus their phenotype and their probable role in response to inflammation.

The interaction of $CD4^+$ T cells with neurons are less characterized in infection because neurons do not express MHC II. Nonetheless, during inflammation Th17 exhibit a preferential ability to engage sustained contacts with neurons (Liu et al., 2015, Egen and Ouyang, 2010) and Th17 and Th1 can regulate innate immune responses in the CNS and enhance bacterial clearance (Holley and Kielian, 2012). Other studies have also described interesting MHCII-independent neuron $CD4^+$ T cell interactions where neurons can directly alter the phenotype and function of activated CD4 T cells to that of regulatory T cells (Liu et al., 2006). Additionally, during CNS injury, neurons release damage-associated molecules that induce a neuroprotective $CD4^+$ T cell response that is independent of MHCII/TCR interactions (Walsh et al., 2015). Together, these studies point towards a complex relationship between neurons and adaptive immune responses within the CNS, with neurons now regarded as important players in directing such responses. There is still lack of complete understanding of interaction between the CNS and the immune system, and mechanisms associated with the role of T cells in neuroprotection and/or neurodegeneration in the CNS. Protective and pathogenic T cell responses occur in response to infection but the

pathway by which antigens are able to exit the CNS to prime T cells in the periphery remains unknown. With new lymphoid structures discovered in association with the brain and now also challenging the pathway of antigen recognition and signal for immune surveillance and response, more research is still needed.

1.5. Study Aims

The role of neurons in the immunological defence against CNS pathogens has always believed to be passive because neurons do not express MHC II. However recent studies have shown that during viral infection neurons express MHC I and interact with CD8 T cells. Another evidence of direct immune-regulatory role of neurons on T cells was observed in the studies of experimental autoimmune encephalomyelitis where neurons can directly alter the phenotype and function of CD4 T cells. Therefore, the aims of this project are:

- To characterize neuronal responses induced by mycobacteria.
- To establish a co-culture model of immune and neural function.
- To investigate the importance of interaction between neurons and immune cells in immunity against mycobacterial challenge.

2. METHODS

2.1. Ethics Statement

Ethical approval was obtained from the Animal Ethics Committee of the University of Cape Town (UCT) – protocol number 015/010. Male and female C57BL/6 mice between 6-8 weeks old were bred and maintained at UCT animal unit under specific pathogen free (SPF) conditions. Mice were housed in filter top cages at the biosafety level 2 facilities at UCT.

2.2. Primary neuron culture

2.2.1. Treating plates with poly-l-lysine

To provide better cell attachment, the day before primary neuron culture preparation, plates were coated with 0.01% poly-l-lysine (Sigma, MO, USA) dissolved in dH_2O and filtered to sterilize. The plate was incubated with 1ml of the poly-l-lysine solution in each well at 4°C for 24 hours. Poly-l-lysine solution was removed and the plate was rinsed thrice with sterile water.

2.2.2. Dissection of the hippocampus

Adult mice were mated with the ratio of three female mice to one male mouse. Pregnant mice were monitored for 17 days and then euthanized with 5% Halothane (Safeline pharmaceuticals). To uncover the foetuses from pregnant female, incision was made along the abdomen of the pregnant female then the uterus was removed. Foetuses were taken out still in their placental sacs, and the sacs were opened without hurting the heads. The umbilical cord was cut then foetuses were removed from the placental sacs. The foetuses were euthanized by decapitation using sharp scissors and heads were collected into 50ml tube containing dissection medium - Hank's balanced salt solution (HBSS) (Lonza, MD, USA) for

transportation to the laminar flow. Under the laminar flow, the skull was opened and brains were suspended in HBSS (Figure 2-1). Hippocampus was chosen for primary neuron cultures as pyramidal neurons, the principal cell type in the hippocampus account for a vast majority of total neuronal population and they express key neuronal phenotypic features in culture (Kaech and Banker, 2006). Meninges-free hippocampi were dissected under the microscope (Figure 2-2) and collected into 15ml tube containing 5ml of HBSS on ice.

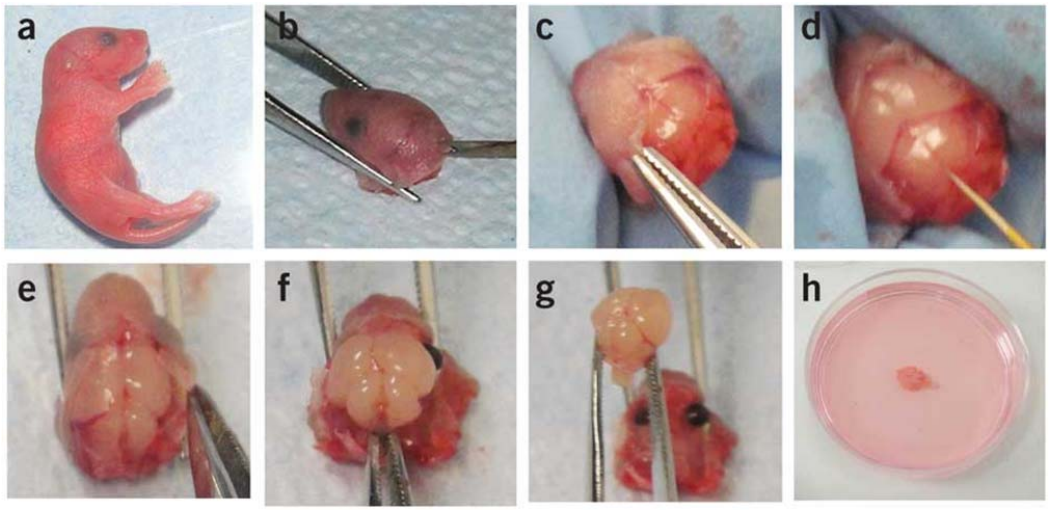


Figure 2-1: Illustration of the technique to remove brains from embryonic day 17 (E17) mouse foetuses. (**a**,**b**) Foetus decapitation and removal of the head using sharp scissors. The skin was cut open from the back to the front to expose the skull. (**c**) Forceps were then used to pull the skin from the skull. (**d,e**) and the skull was also removed, revealing the brain. (**f**) Forceps were gently placed underneath the brain and separated it from underlying tissue. (**g**,**h**) The brain was gently scooped out and quickly submerged into dissection medium (HBSS [Lonza]). Source: (Beaudoin et al., 2012)

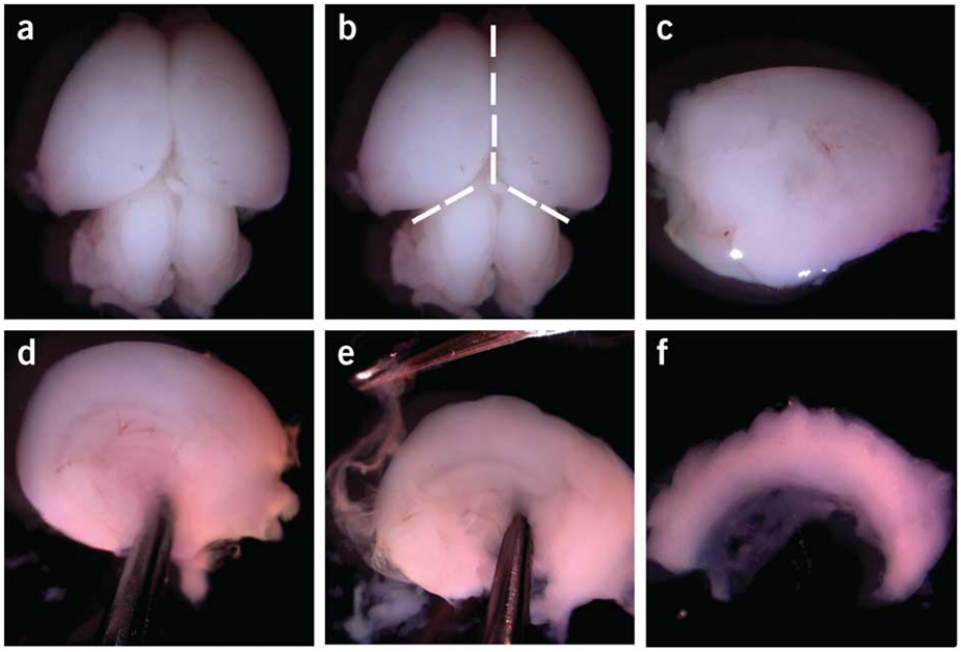


Figure 2-2: Illustration of the dissection of the hippocampus from the mice foetal brain. (a) The brain was placed with dorsal side up in HBSS. (b) The hindbrain region and the two hemispheres were separated by indicated incisions. (c) The hemisphere was placed side down and forebrain tissue was removed. (d) The hemisphere was gently held in place to avoid damage to the hippocampus. (e) The meningeal tissue was removed with another pair of forceps. (f) Hippocampus was dissected out. Source: (Beaudoin et al., 2012)

2.2.3. Cell dissociation and plating

The tissue was allowed to settle before the HBSS was removed and 500µl – 1ml of Papain digestion buffer (0.1% papain [Sigma, MO, USA], 0.02% BSA [Roche, Mannheim, Germany] in neurobasal medium [Gibco, NY, USA]) was added and incubated in a 37°C water bath for 10 minutes. The supernatant was carefully removed and to wash residual papain, 5ml of Neurobasal medium (Gibco) was added and tube was left to stand at room temperature (RT) for tissues to settle before removing the supernatant and this was repeated twice. 1ml of Plating neurobasal medium (2% B27 supplement [Gibco, NY, USA], 1% L-glutamine [Gibco, NY, USA] in neurobasal medium [Gibco]) was added and tissues were dissociated by trituration using P1000 pipette. Once homogenous suspension was achieved,

10µl aliquot of dissociated cells was taken to determine viable cell density using haemocytometer. 10µl of Trypan Blue (0.4% trypan blue solution [Sigma]) was added to 10µl of cells and viable cells were identified by their light glossy appearance. To calculate number of viable cells, average cell count was multiplied by 2 from trypan blue addition, and then multiplied by 10^4.

Number of cells to be seeded was determined based on the type of plate used (Table 2-1). These provided sufficient cell density for neuronal cell development to confluency. Cells were added to 48 well plate for neuron T cell cell-to-cell contact experiments, or 24 well plate for transwell - contact independent experiments (Table 2-1). Plates were sealed with micro-pore tape and incubated at 37°C with 5% carbon dioxide (CO_2) until confluency (Figure 2-3). Media were partly changed every 3-4 days using plating neurobasal medium warmed in 37°C water bath.

Table 2-1: Seeding densities of primary neuronal cultures

Culture type	Plate type	Experiments/ Applications	Well area	Number of cells to plate per well	Neurobasal medium final volume/ well
Primary neurons	48 wells	- neuron T cell direct contact co-culture	0.95 cm^2	$3x10^5$	300ul
	24 wells	-transwell -ICC	1.91 cm^2	$4x10^5$	500ul

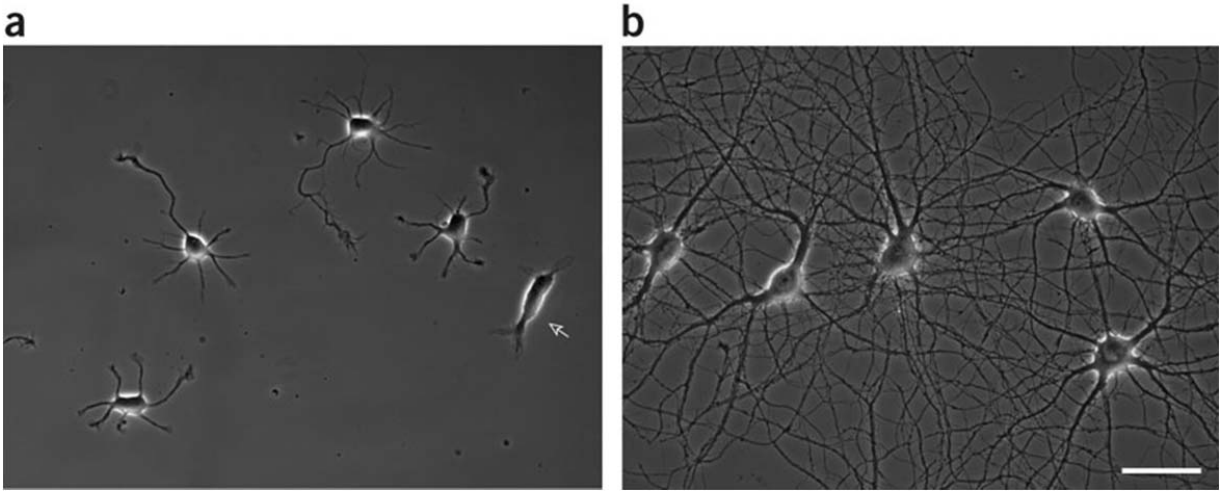


Figure 2-3: Phase contrast images of hippocampal neuron cultures after 1 day (a) and 13 days in culture. Source: (Kaech and Banker, 2006).

2.3. Infection of Primary Neurons

2.3.1. Mtb Strain H37RV/ H37Rv-GFP and BCG-GFP Stock Preparation

H37Rv/ H37Rv-GFP (kindly provided by Joel Ernst, New York University School of Medicine, New York, USA) and BCG-GFP were cultured for 21 days in Difco™ Middlebrook 7H9 broth (Becton) containing 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC) (BD Biosciences, MD, USA) and 50µl/ml Kanamycin (Santa Cruz, CA, USA) or Hygromycin B (Roche) and incubated at 37°C, 5% CO_2. The mycobacteria without GFP expression (H37Rv) were cultivated in the absence of antibiotics. 1ml aliquots were prepared in sterile conditions and stored at -80°C in 2ml screw cap vials (Greiner bio-one, Cryo Cellstar). To determine viable Mtb concentrations of the frozen stocks, 10-fold serial dilutions were made and plated on Difco™ Middlebrook 7H10 agar (Becton) plates containing 10% OADC, 50 µl/ml Hygromycin B or Kanamycin and incubated at 37°C, 5% CO_2 for 21 days. The final viable Mtb concentration was calculated based on the number of colonies counted.

2.3.2. Inoculation of H37Rv and BCG

BCG and H37Rv stocks were stored at -80°C freezers in biosafety level 2 (BSL2) and biosafety level 3 (BSL3) laboratory respectively. Mycobacteria were thawed at RT, centrifuged at 10000rpm at 4°C for 10 minutes to remove glycerol. The pellet was resuspended in plating neurobasal medium. To reduce clumping, the BCG suspension was passed through a 29-gauge needle 30 times. On the other hand, due to the health and safety reason, no sharps was allowed in BSL3 laboratory, therefore the H37Rv suspension was transferred into tubes containing 2-3 mm glass beads and vortexed vigorously for one minute. Primary neurons were infected at multiplicity of infection (MOI) of 30:1. The plate was sealed with micro-pore tape and incubated at 37°C, 5% CO_2 for 24 hours of which optimal infectivity can be achieved as previously shown (Randall et al., 2014).

2.4. Co-culture with T Lymphocytes

To obtain naïve T Cells, spleens were collected from naïve 6-8 weeks old C57BL/6 mice into 10 ml 1X PBS (0.137M NaCl, 2mM KH_2PO_4, 3mM KCl, 8mM $Na_2HPO_42H_2O$) on ice. For single cell suspension – spleens were pressed through 70-micron sieve using a syringe plunger, and cells were collected by centrifugation for 5 minutes at 1200rpm at 4°C. Supernatant was discarded, the pellet was resuspended in 1-2ml of Red blood cell lysing buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1M Na_2EDTA) and left to stand for 1-2 minutes at RT. Following neutralization and centrifugation, supernatant was discarded. The cells were incubated with CD3 antibody (25µl to 10^6 cells) (Table A-1) for 20 minutes at 4°C then washed with FACS buffer. Supernatant was discarded and the pellet was resuspended in 5ml of neurobasal medium and filtered through 0.22µm FACS tube filter to remove remaining clumps. $CD3^+$ T Cells were sorted using Fluorescent Activated Cell Sorting (FACS) (BD

FACSAria) into 15ml falcon tubes containing 2ml neurobasal medium (Figure A-1). Due to technical issues of the cell sorter, in BCG experiments, the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell populations were obtained by manual sort using the Miltenyi Biotec Magnetic Activated Cell Sorting (MACS) bead system according to manufacturer guidelines https://www.miltenyibiotec.com/~/media/Images/Products/Import/0001900/IM0001983.ashx?force=1. To determine viable T cell density, 10µl aliquot of sorted cells was taken, added to 10µl trypan blue and density of cells was determined on a haemocytometer. The T cells were added to neuronal cultures at a ratio of 1:1 after 24 hours of neuronal infection. For neuron and T cell cell-to-cell contact, T cells were added directly neurons and the effects of this contact were evaluated against cultures where neurons and T cells were separated by a transwell membrane. To study the effects of infected neuron on T cells in the absence of possible contact or communication, neuron conditioned media was added to T cells. The co-cultures were incubated for 48 hours at 37°C, 5% CO_2 as it was previously shown (Randall et al., 2014) that neurons induce an immune response after 48 hours *Mtb* infection.

2.5. Flow Cytometry (FACS)

Flow cytometry was used to analyse the expression of cell surface proteins and intracellular proteins using florescent labelled monoclonal antibodies. To prepare cells for FACS staining, the culture plates were centrifuged to settle floating cells and supernatants were collected for ELISA analysis. Adhered cells were lifted using 200µl/well of 1X Trypsin EDTA (10X Trypsin EDTA [Gibco] diluted in HBSS [Lonza]) and incubated for 5 minutes at 37°C, 5% CO_2. To neutralise trypsin reaction, equivalent volume of plating neurobasal medium was added. For each co-culture well, the volume was halved for staining both the neuronal antibody panel (Table A-2) and T cell antibody panel (Table A-1).

2.5.1. FACS extracellular staining

Lifted Cells were transferred to a 96 well plate and then washed with FACS buffer (0.1% BSA, 0.01% NaN_3 in 1X PBS, pH 7.4). For staining, 25µl/well (25µl to 10^6 cells) of Ab cocktail for each panel was added and incubated at 4°C for 20 minutes. Cells were then washed with FACS buffer, fixed in fixation buffer (2% paraformaldehyde in 1X PBS, 4% NaOH in dH_2O, pH 7.2) and stored at 4°C before intracellular staining.

2.5.2. FACS intracellular staining

2.5.2.1. Neuron Panel

For intracellular staining of the cells, fixation buffer was removed and 200µl/well FACS buffer was added to wash. Then 200µl/well of permeabilization buffer (1% saponin in FACS buffer) was added for 30 minutes at 4°C. Centrifuged for 10 minutes, 1200rpm, 4°C and discarded supernatant. 25µl/well of intracellular Ab cocktail (Table A-2) was added and incubated for 20 minutes then washed with 200µl/well permeabilization buffer. Cells were kept in 200µl/well fixation buffer and stored at 4°C until flow cytometry acquisition. Analysis of primary neurons was based on gating in figure A-2.

2.5.2.2. T cell transcription factors panel

Intracellular staining of T cell transcription factors was performed according to BD Biosciences transcription factors (Table A-1) staining protocol http://www.bdbiosciences.com/ds/pm/tds/562725.pdf. Stained T cells were stored in 200µl/well of FACS buffer at 4°C until flow cytometry acquisition. Analysis of T cells was based on gating in figure A-3.

Acquisition was done on BD LSRFortessa and data analysis of FCS files was done using FlowJo version 9.8.5.

2.6. Enzyme-linked Immunosorbent assay (ELISA)

ELISA was used to detect and quantify cytokines of interest in Table A-3. Supernatants collected after 48 hours of co-culture were analysed by ELISA. The plate was centrifuged for 5 minutes, 800rpm, 4°C to settle floating cells and supernatants were collected and filtered using a 0.22µm filter (Merck Millipore) and lure lock syringe into sterile Eppendorf (UCT BSL3 facility requirements) and stored at -80°C. To coat the ELISA plate, capture antibody was diluted appropriately (Table A-3) in dilution buffer (1% BSA, 0.02% NaN3 in 1X PBS) and maxisorb nunc 96 well plate (Nalge, Nunc International, USA) was coated with 50µl/well of the anti-cytokine antibody and left overnight at 4°C. Plate was washed 4 times with washing buffer (13.4mM KCl, 5.8mM KH2HPO4.2H2O, 700mM NaCl, and 2.03mM Tween 20). It was then blocked with 200µl/well blocking buffer (4% BSA, 0.02% NaN3 in 1X PBS), left at 4°C overnight or incubated at 37◦C for 2 hours and washed 4 times. 50µl/well recombinant cytokine standards (Table A-3) and samples were added and plates were incubated overnight at 4°C or for 2 hours at 37°C. 50µl/well of Biotinylated Secondary Ab (Table A-3) was added and incubated for 1 hour at 37◦C. The plate was washed 4 times with washing buffer. Streptavidin alkaline phosphatase (BD Pharmigen^{TM}) was diluted 1:1000 in dilution buffer (1% BSA, 0.02% NaN3 in 1X PBS) and 50µl/well was added, incubated again for 1 hour at 37°C and washed the plate 4 times. Substrate buffer prepared at a dilution of 0.05g 4-nitrophenyl disodium salt-hexahydrate in 50ml substrate buffer (0.02% NaN3, 1.04M Di-ethanolamine, 3.9mM MgCl2.6H2O, pH 9.8 with 10M HCL) and 50µl/well was added. The reaction was allowed to develop and the plate was read at 405nm (492nm

reference wavelength) on an ELISA reader (Molecular Devices, Spectra MaxGemini, USA) using Soft Max programme.

2.7. In vivo – intracerebral H37Rv/BCG infection

Four to five C57BL/6 female mice were inoculated intra-cerebrally in the left cerebral hemisphere with $1x10^5$ CFU of H37Rv or BCG-GFP using Hamilton syringe. For H37Rv infection experiments, mice were euthanized at 7, 14 and 21 days' post infection. Brains were remove from the skulls and were pressed through 70-micron sieve for single cell suspension. To determine the number of viable cells to be stained, 10µl of cells were added to 10µl of trypan blue and cell number was determined using a haemocytometer. Extracellular and intracellular staining protocols were followed as previously outlined (2.5.1 & 2.5.2) and the antibodies used for staining are shown in Table A-4. BD LSRFortessa was used for acquisition and data analysis was done using FlowJo version 9.8.5. Analysis of neurons was based on gating strategy illustrated in Figure A-4.

2.8. Statistical Analysis

Statistics were performed with GraphPad Prism 6 statistical software. Statistical methods used were Student unpaired t-test (Two-tailed), one-way ANOVA with Bonferroni's post test and 2way ANOVA with Bonferroni's post test. A P value of less than 0.05 was considered significant.

3. RESULTS

3.1. Neuronal mycobacterium tuberculosis uptake and response

3.1.1. Primary neurons internalize Mtb in vitro

The causative agent of TB, *Mycobacterium tuberculosis*, preferentially infects macrophages in pulmonary infection and microglia in CNS infection. It is now known that Mtb can also infect a variety of cells including "non-classical" immune cells such as epithelial cells and fibroblasts (Randall et al., 2015). A novel observation was previously reported that neurons were infected by Mtb during *in vitro* and *in vivo* infection (Randall et al., 2014). To further characterize whether the pathogenesis of neuronal infection depends on the virulence of the bacteria, primary neuronal cultures were infected with either virulent (H37Rv) or avirulent (BCG) strain of mycobacteria. Primary neurons cultivated from hippocampus of embryonic day 17 (E17) C57BL/6 mice (2.3.2) were infected with a recombinant GFP expressing H37Rv (Mtb laboratory strain) at multiplicity of infection (MOI) of 30:1 for 24 hours. Similarly, primary neurons were also infected with Bacillus Calmette–Guérin expressing GFP (BCG-GFP) (2.4.2). Post infection, cultured neurons were stained with β-III-tubulin antibody, a neuronal marker and analysed by flow cytometry. β-III-tubulin^{+} neurons containing GFP^{+} bacilli were detected in both H37Rv-GFP and BCG-GFP infected cultures (Figure 3-1). The percentage of neurons infected by H37Rv-GFP was 13% (Figure 3-1 A), which falls in the same range as previously reported (Randall et al., 2014). On the other hand, the flow cytometry analysis revealed that the percentage of βtubulin^{+} GFP^{+} neurons was 5% in the BCG-GFP infected cultures (Figure 3-1 B). Although the percentage of BCG-GFP infection was lower than the H37Rv-GFP infection, this is the first evidence to suggest direct interaction between neurons and BCG.

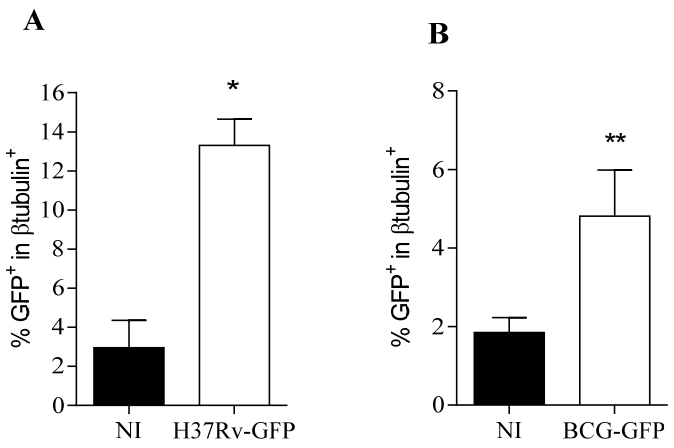


Figure 3-1: Flowcytometry analysis of β-III-tubulin$^+$ GFP$^+$ primary neurons cultures infected with (A) H37Rv-GFP and (B) BCG-GFP for 24 hours. Neuron cultures were infected at MOI of 30:1 and results represent percentage of primary neurons that internalized Mtb bacilli (GFP and β-III-tubulin double positive cells). 13% of cells in Mtb infected neuron cultures have detectable expression of GFP and a lesser percentage (5%) of cells expressed GFP in BCG-GFP infected cultures. Data are mean ± SD. *P < 0.05, **P < 0.01. NI – non-infected

3.1.2. How do neurons respond to mycobacterial infection?

Neurons are shown to be activated during Mtb infection (Randall et al., 2014). However, the impact of mycobacterial infection on neuronal immune reaction is not clear. To address that, primary neurons were grown in culture until confluency (2.3.3) and infected with H37Rv at MOI of 30:1 for 24 hours (2.4.2). Infected cultures were compared with non-infected cultures. To assess phenotypic changes activated through neuronal interaction with Mtb, the expression of surface markers associated with cellular or neuronal response to stimuli were examined. Post infection, primary neurons were stained using fluorescent labelled monoclonal antibodies for flow cytometry analysis. Interaction of primary neurons with live Mtb resulted in cell activation reflected by changes in neuronal surface expression (Figure 3-2) and cytokine expression (Figure 3-3). Upon infection, the frequency of MHC I expression on neurons was found to be significantly higher than the non-infected (Figue 3-2 A).

Mycobacterial stimuli upregulated neuronal expression of MHC I, the antigen presenting molecule, suggesting that infected neurons may have an increased ability to stimulate T cells, particularly $CD8^+$ T cells against mycobacterial antigens. MHC II was undetectable in infected and non-infected neuron cultures which is to be expected as reports show neurons do not express the surface protein (Figure 3-2 B).

To continue analysis of neuronal immune related functions activated through cells interaction with Mtb, the expression of selected cytokines was measured. The cytokine panel chosen was guided by various reports in viral and bacterial CNS infections where induction of neuronal cytokines such as IL1β, TNF and IL10 was observed. The expression levels of these cytokines in infected neuronal cultures were determined by intracellular staining for flow cytometry analysis. Mycobacterial infection significantly upregulated the expression of IL1β in neurons (Figure 3-2 C). There was no significant difference in neuronal expression of TNF and IL10 in infected and non-infected cultures (Figure 3-3 D, E). In summary, mycobacterial infection of neuronal cultures induces neuron activation which includes upregulation of MHC I and inflammatory cytokines that are essential to the resolution of mycobacterial infections.

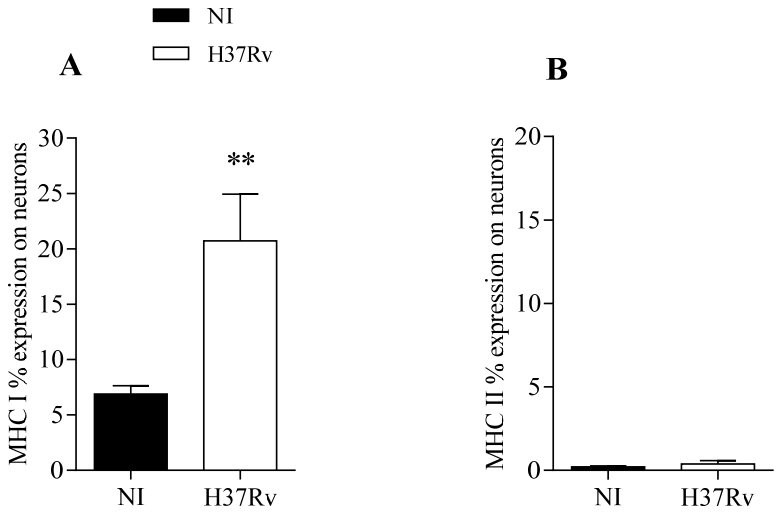


Figure 3-2: Cell surface expression of neurons after infection with mycobacteria. Results represents flow cytometry analysis of the percentage of β-III-tubulin^{+} primary neurons expressing (A) MHC class I and (B) MHC class II measured in H37Rv infected cultures and non-infected controls. Data are mean ± SD. *P < 0.05, **P < 0.01. NI – non-infected

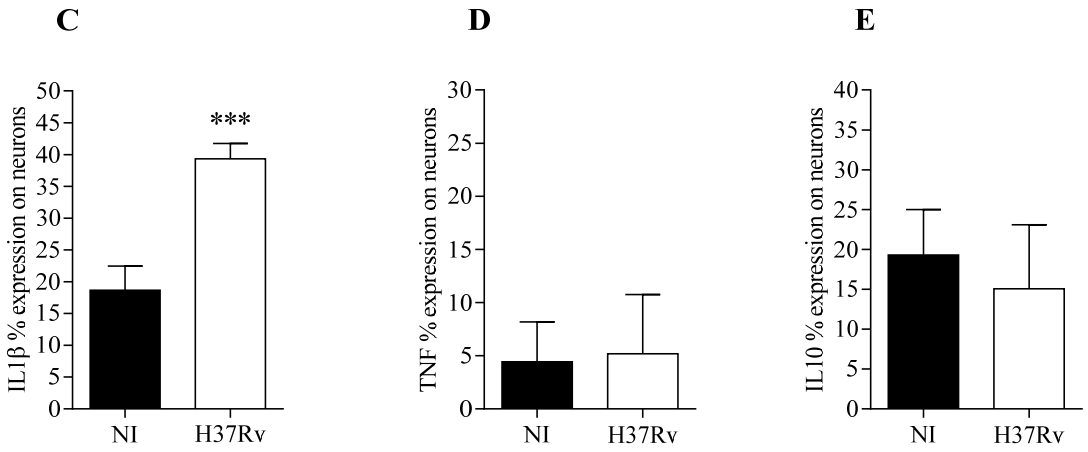


Figure 3-3 Cytokine expressions of primary neurons after infection with Mtb. Neurons were infected with H37Rv (MOI = 30:1) for 24 hours and analysed by flow cytometry for cytokine productions of (A) IL1β, (B) TNF and (C) IL10. The results represent percentages of β-III-tubulin^{+} neurons expressing cytokines in infected cultures and non-infected controls. Data are mean ± SD. *P < 0.05, **P < 0.01. NI – non-infected

3.2. Neuronal response to mycobacterial infection in the presence of T cells

As previously described, neuronal expression of MHC class I and inflammatory cytokines were upregulated under infectious conditions that suggests potential interaction of neurons with T cell may influence disease outcome. So a co-culture model was used to address the effect of T cells on neuronal inflammation or neuronal response to Mtb. Neuron T cell co-culture was established by isolating splenic T cells from mice via cell sorting using FACS. $CD3^+$ T cells were sorted and co-cultured with H37Rv infected neuron cultures (2.5). Flow cytometry was used for analysis of neuronal surface expression of MHC class I and II, as well as cytokines, IL1β, TNF and IL10, in neuron only cultures and neurons co-cultured with $CD3^+$ T cells. The percentage of neurons expressing MHC class I was significantly decreased upon co-culture with T cells when comparing between infected neuron only cultures and neuron T cell co-cultures. No significant difference was observed in MHC class I expression in non-infected neuron with or without T cells (Figure 3-4 A). Addition of T cells had on effect on levels of neuronal MHC class II expression (Figure 3-4 B). Presence of T cells also resulted in reduction in IL1β expression in neurons (Figure 3-4 C). No significant changes were observed in TNF and IL10 expression levels (Figure 3-4 D, E). The secretion of cytokines in the supernatants were analysed by ELISA for the amounts of IL6 and IL10 in neuron only culture and neuron T cell co-cultures. To study the effects of cell-to-cell contact on neuronal response, the concentrations of cytokines from the direct co-culture system were compared to that of the transwell system. Production of IL6 was significantly increased in H37Rv infected neuron only culture at a mean concentration of 0.24 ng/ml as compared to 0.03 ng/ml in non-infected cultures. However, no changes were observed in IL6 production in both direct and transwell co-cultures (Figure 3-5 A). Mycobacterial infection did not have any effect on the production of IL10 in neuron only cultures. There was a significant difference in IL10

production in direct neuron T cell co-culture which was not observed in transwell co-cultures (Figure 3-5 B).

These data suggest that T cells are able to mediate MHC class I and IL1β expression in the Mtb infected neurons. The secretion of IL10 in neuron T cell co-culture is contact dependant during Mtb infection.

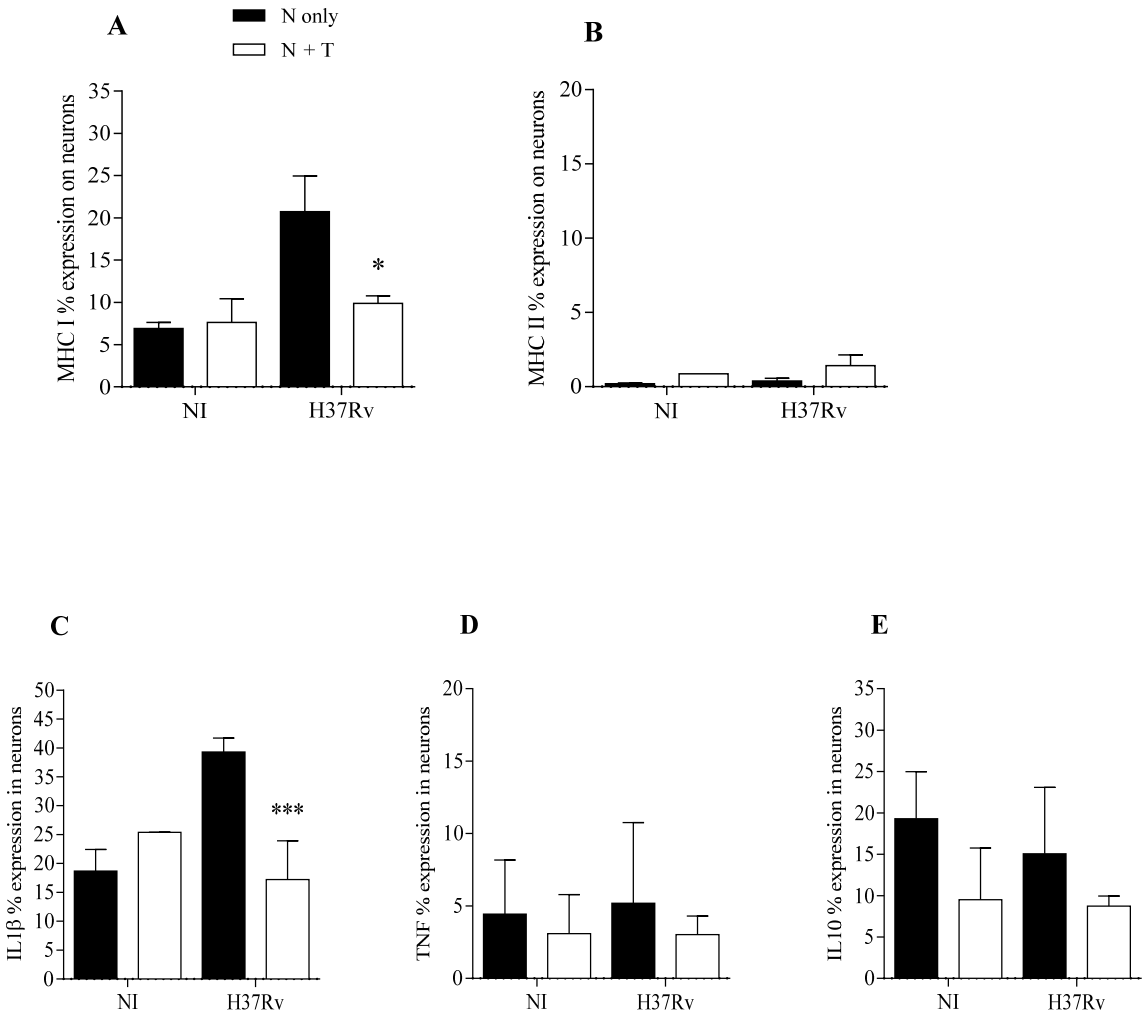


Figure 3-4: Primary neurons were infected with H37Rv, MOI = 30:1 for 24 hours. Infected neurons cultures and non-infected controls were co-cultured with splenic $CD3^+$ T cells at a ratio of 1:1 for 48 hours. FACS was used for analysis of neuronal surface expression of A) MHC class

I, B) MHC class II and intracellular expression of C) IL1β, D) TNF and E) IL10 in infected and non-infected neuron cultures. The graph indicates the percentage of β-III-tubulin^{+} neurons expressing the surface proteins MHC class I, MHC class II and inflammatory cytokines (IL1β, TNF and IL10) in neuron only culture and neurons co-cultured with T cells. N – neurons, T – T cells. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001

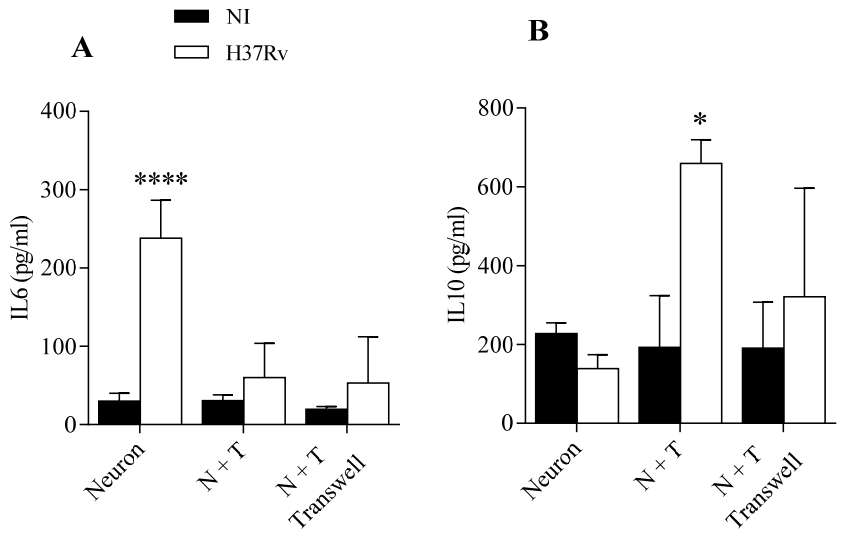


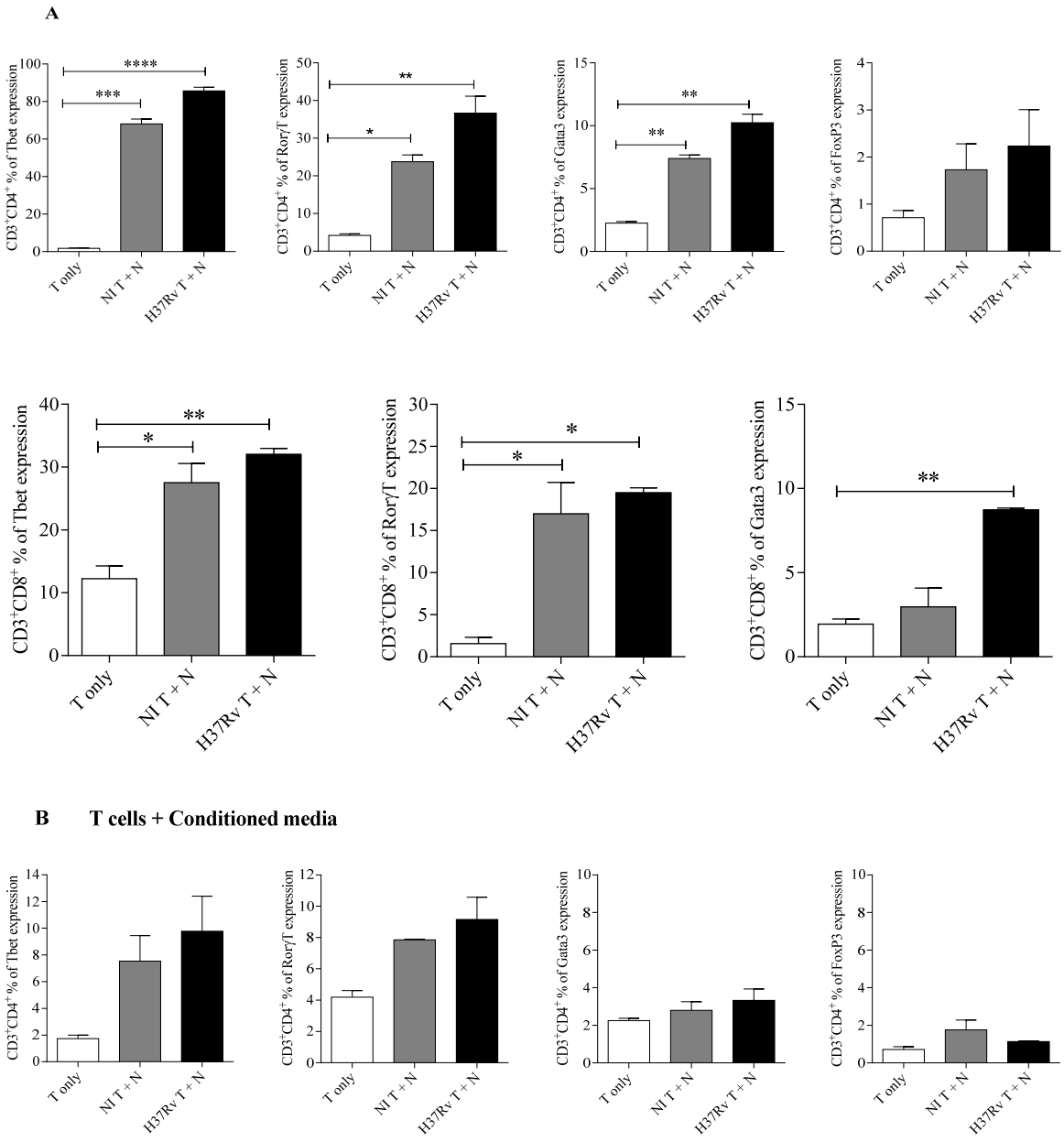
Figure 3-5: The concentrations of IL6 and IL10 secreted in culture supernatants by infected and non-infected neurons with and without T cells analysed using ELISA. Neurons were infected with Mtb for 24 hours and incubated 48 hours with T cells, in either direct or indirect (transwell) co-cultures systems. NI–non-infected, N-neurons, T – T cells. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

3.3. Role of neurons in mediating T cell responses

The role of neurons in regulation of T cell responses is well studied in CNS autoimmune and neurodegenerative disorders. Considering the critical role of T cells in resolution of infectious pathogens, the study sought to characterise the neuron mediated T cell responses in mycobacterial infection in vitro. The effects of neuron T cell interaction on T cell differentiation were determined using a co-culture model. Cells were collected after 48hours of co-culture and expressional levels of T cells specific transcription factors, Tbet, FoxP3,

Gata3 and RorγT, were analysed by flow cytometry. Analysis was performed on $CD3^+CD4^+$ and $CD3^+CD8^+$ T lymphocytes (Figure A-3). There was a significant increase in the percentage of $CD4^+$ and $CD8^+$ T cells expressing Tbet in the presence of neurons (Figure 3-6 A). The upregulation of Tbet was observed in co-cultures of T cells with infected and non-infected neurons suggesting that independent of infection, the direct interaction with neurons is sufficient to drive a Th1 response in $CD4^+$ and $CD8^+$ T cells. Similar results were observed in the expression of RorγT and Gata3 (Figure 3-6 A).

The effects of cell to cell contact responses were evaluated against transwell system co-cultures where neurons and T cells were separated by a membrane and a co-culture of T cells with neuron conditioned media. $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells were analysed by flow cytometry for the T cell transcription factors. There was no significant difference in the percentage of $CD4^+$ T cells expressing Tbet, RorγT, Gata3 and FoxP3 (Figure 3-6 B). However, there was difference in the $CD8^+$ T cells expressing RorγT in cultures of T cells with infected and non-infected neuronal media. No changes were observed in the percentage of $CD8^+$ T cells expressing Tbet and Gata3 (Figure 3-6 B). Through transwell separation, neurons were still able to upregulate expression of Tbet, RorγT, Gata3 and FoxP3 in $CD4^+$ T cells (Figure 3-6 C). In ELISA analysis of supernatants, IFN-γ and IL10 production was undetectable in supernatants of T cells only cultures and increased in culture supernatants of neurons co-cultures directly with $CD3^+$ T cells. There was difference in the production of both cytokines in infected and non-infected transwell system co-cultures (Figure 3-7). These data demonstrate direct contact between neurons and T cells plays a significant role in T cell transcription factors expression and probable T cell activation in vitro.


A
CD3+CD4+ % of Tbet expression
CD3+CD4+ % of Ror/T expression
CD3+CD4+ % of Gata3 expression
CD3+CD4+ % of FoxP3 expression
CD3+CD8+ % of Tbet expression
CD3+CD8+ % of Ror/T expression
CD3+CD8+ % of Gata3 expression
T only
NI T + N
H37Rv T + N
B T cells + Conditioned media
CD3+CD4+ % of Tbet expression
CD3+CD4+ % of Ror/T expression
CD3+CD4+ % of Gata3 expression
CD3+CD4+ % of FoxP3 expression
T only
NI T + N
H37Rv T + N

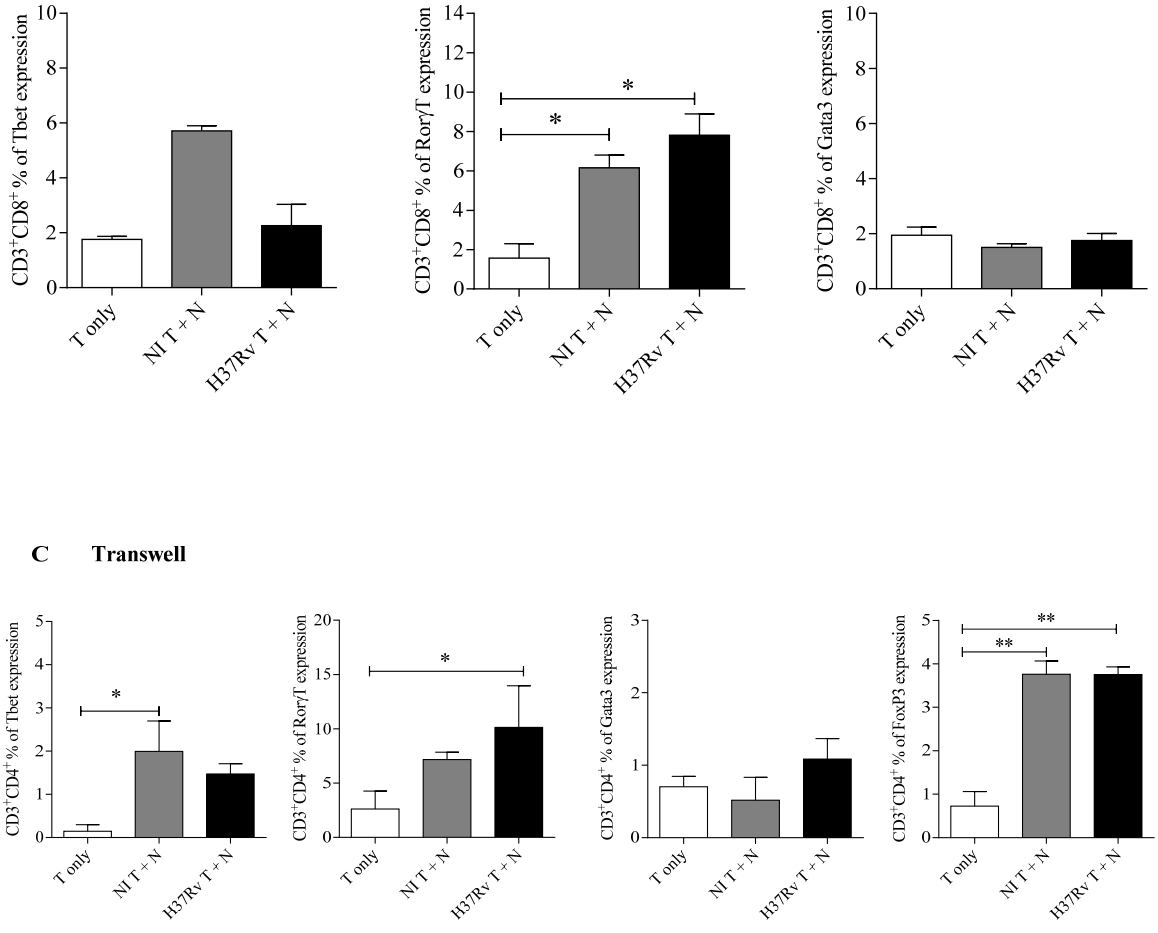


Figure 3-6: A) These data represents FACS analysis of the percentage of $CD3^+CD4^+$ and $C3^+CD8^+$ T cells expressing transcription factors in T cell only cultures (white bar) and T cells co-cultured with neurons (grey bar - non infected; black bar – H37Rv infected) at a ratio of 1:1 for 48 hours B) Transcription factors expression in sorted T cells after with neuron culture conditioned media. C) FACS analysis neuron T cell co-culture where direct contact was prevented using a transwell system and T cells only served a control. N – neurons, T – T cells. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

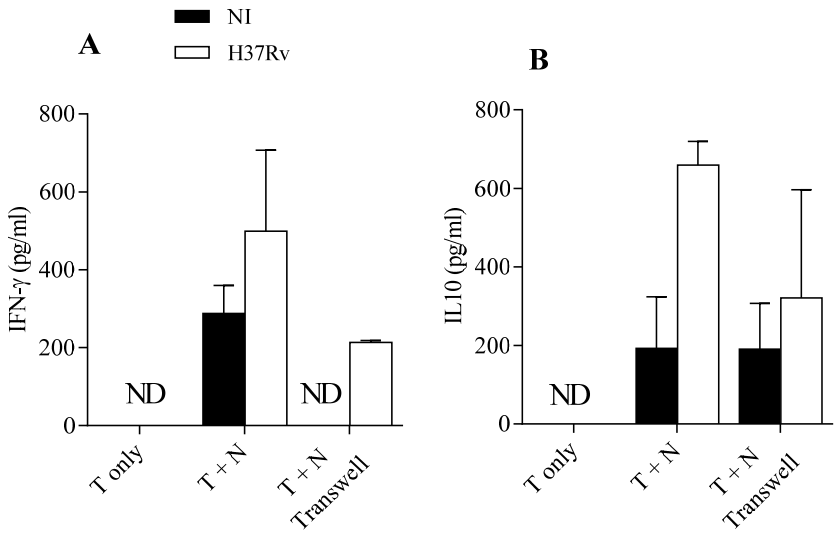
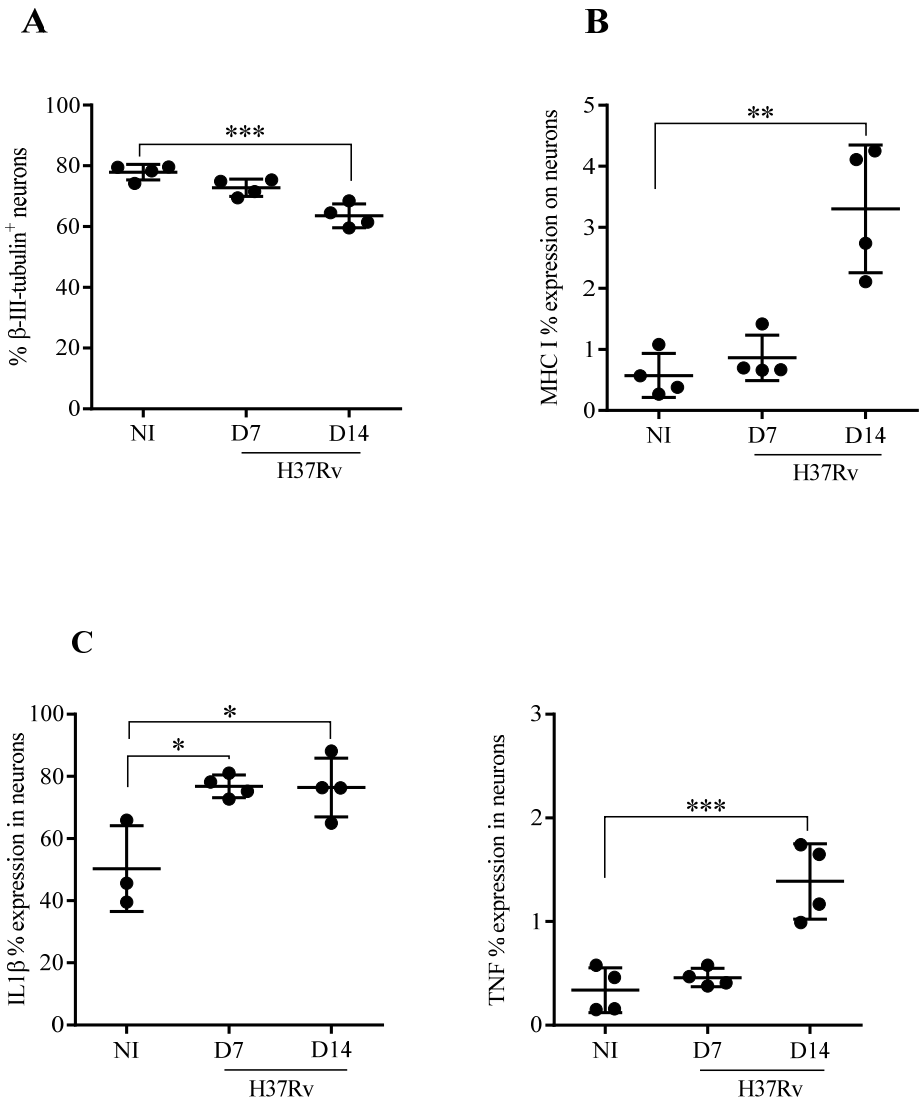


Figure 3-7: IFN-γ and IL10 production in culture supernatants of T cells and T cells co-culture with neurons directly or through a transwell system assessed by ELISA. N – neurons, T – T cells. Data are mean ± SD. ND- non detectable.

3.4. Effects of mycobacterial infection on neuronal phenotype and function in vivo

Neurons internalize Mtb bacilli in vivo 7 days after infection (Randall et al., 2014) and whether they respond to the internalization and presence of mycobacteria in vivo remains unclear. To address this, adult C5BL/6 mice were infected intracerebrally with Mtb (H37Rv strain) (2.8). The activation status of neurons was analysed by flow cytometry for a selection of cell surface markers and expression of inflammatory cytokines. Neurons were defined as β-III-tubulin$^+$ cells for FACS analysis and evaluated from whole brains collected at different time points post infection. A significant reduction in β-III-tubulin$^+$ neurons percentage between non-infected and day 14 of infection was observed (Figure 3-8 A). Analysis of neuronal MHC class I revealed a marked in vivo activation of β-III-tubulin$^+$ neurons (Figure 3-8 B) which gradually increased over the course of infection and peaked at day 14.

Cytokines (IL1 β, IL6, TNF an IL10) expression was also assessed due to their well-documented key roles in response to mycobacterial infection. Flow cytometry analysis of these molecules, except IL10, showed their expression increased significantly following the same kinetics as MHC class I (Figure 3-8 C). Results indicate substantial activation of neurons demonstrated by an increase in MHC class I expression and inflammatory response in vivo during acute mycobacterial infection.



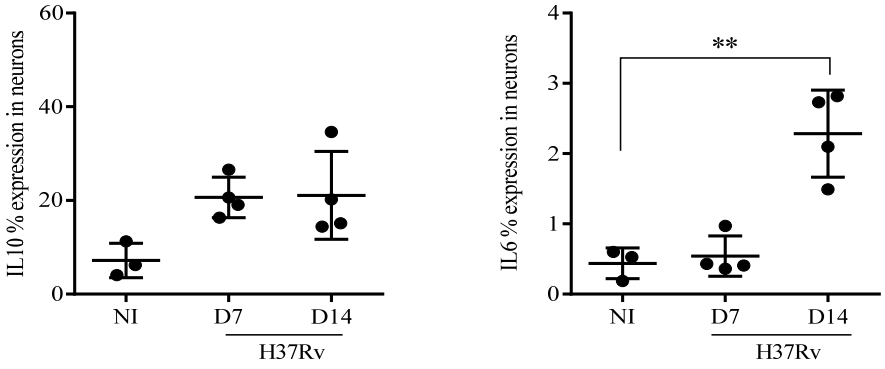


Figure 3-8: Neurons from whole brains of (NI, *n=4*) Mtb infected C57BL/6 mice at day 7 (*n=4*) and day 14 (*n=4*) analysed by flow cytometry by gating on β-III-tubulin^{+} neuronal cells. A) Comparison in percentages of acquired neurons between 7 and 14 days of infection. B) Percentage of MHC class I expression by neurons. C) IL1β, IL6, TNF and IL10 expression by neurons gated on β-III-tubulin^{+} cells and was analysed by flow cytometry. NI – non-infected, D7 – day 7, D14 – day 14. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

3.5. Neuronal response to infection by less pathogenic mycobacteria (BCG-GFP)

In the initial experiments (3.1.1) it was shown that neurons can internalize BCG. To assess neuronal response to BCG, cells were infected (MOI = 30:1) for 24 hours and stained with β-III-tubulin antibody (neuronal marker) for flow cytometry analysis of selected of cell surface molecules and cytokines. Neuron T cell interactions were studied using a co-culture model of primary neurons and splenic T cells sorted by $CD4^+$ and $CD8^+$ using MACS beads sorting system. The two populations of T cells were co-cultured with BCG infected and non-infected neurons at a ratio of 1:1 for 48 hours. Flow cytometry was used to determine the percentage of neurons expressing MHC class I, IL1β, TNF and IL10 in neuron only cultures and co-cultures of neurons with $CD4^+$ or $CD8^+$ T cells. For control, $CD4^+$ or $CD8^+$ T cells were cultured alone. To analyse direct cell-to-cell contact effects, a co-culture was also established using a transwell system (neurons and sorted $CD4^+$ T cells separated by a membrane). BCG infection upregulated neuronal expressions of MHC I in neuron only cultures with no significant in co-culture samples (Figure 3-9 A). Infection and the T cells did not have any effect on MHC class II expression (Figure 3-9 B). IL1β was significantly increased in BCG-GFP infected neuron cultures. No change was observed in the percentage of $CD8^+$ T cell co-culture but there was significant decrease in $CD4^+$ T cells in direct and transwell co-cultures (Figure 3-9 C). Similar reduction of IL10 was also observed in the $CD4^+$ T cells transwell co-culture post BCG-GFP infection, but overall, there was no significant difference in the expression of TNF and IL10 amongst other groups (Figure 3-9 D, E).

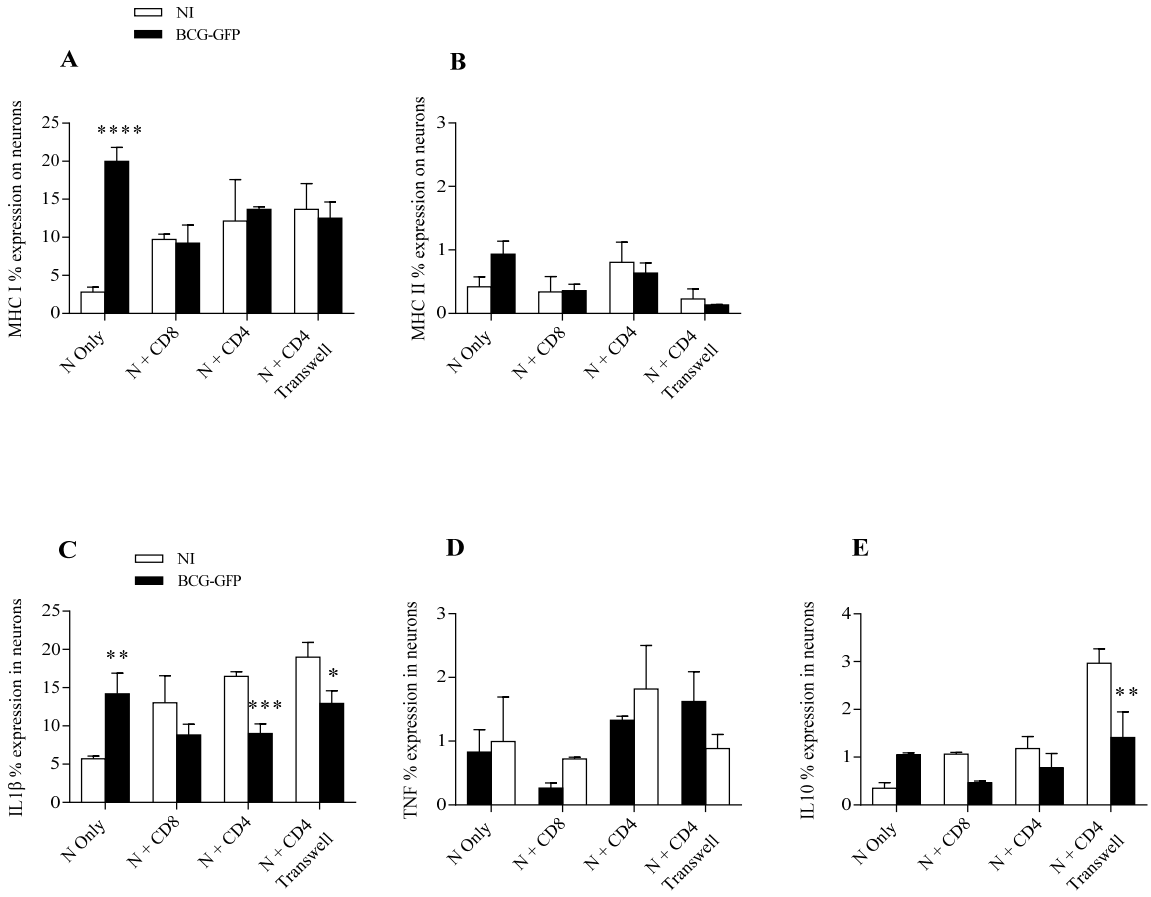


Figure 3-9: Neuronal expression of MHC I, MHC II, IL1β, TNF and IL10 analysed by flow cytometry before and after co-culture with $CD4^+$ and $CD8^+$ T cells (sorted from spleens of wild type C57BL/6 adult mice) in the presence and absence of infection. Neurons were infected with BCG-GFP at MOI=30 for 24 hours then incubated with T cells at a ratio of 1:1 for 48 hours. NI – non-infected, N – neuron. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

3.6. T cell responses to neuron T cell interaction

To study neuronal role in mediating T cell response during BCG-GFP infection, cells in $CD4^+$ and $CD8^+$ co-cultures were stained with T cell transcription factors Tbet, RorγT, Gata3

and FoxP3. Flow cytometry was used to analyse the percentage of $CD8^+$ and $CD4^+$ T cells expressing selected transcription factors. There was a significant difference in the percentage of $CD8^+$ and $CD4^+$ T cells expressing Tbet in T cell only and non-infected co-cultures (Figure 3-10 A). Increase in Gata3 expression was observed between $CD8^+$ only T cell and non-infected neurons co-cultures with $CD8^+$ T cells and a decrease in comparison between infected and non-infected co-culture (Figure 3-10 C). There was no difference in the expression of RorγT (Figure 3-9 B) and FoxP3 (Figure 3-9 D). Therefore, BCG-GFP infection and the presence of neurons mediate the expression of Tbet and Gata3 in T cells.

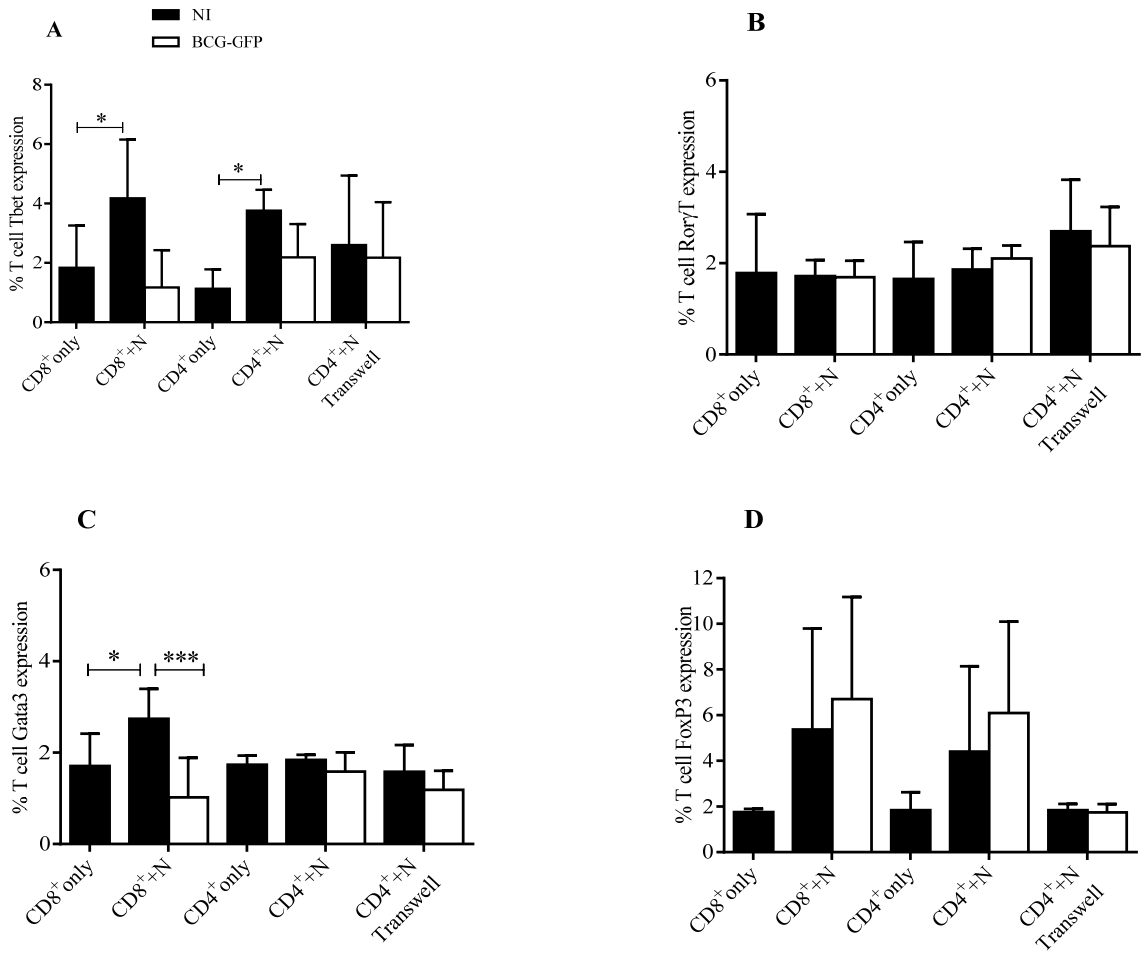


Figure 3-10: T cell expression of transcription factors detected by flow cytometry in T cells and T cells co-cultured with neurons. NI – non-infected, N – neuron. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

3.7. Neuronal response to BCG-GFP infection in vivo

To investigate the neuronal responses to BCG infection in vivo, adult C57BL/6 mice were infected with BCG-GFP by intracerebral infection and whole brains were collected at 3 and 6 weeks of infection. Single cell suspension was performed and $1x10^6$ cells were stained for analysis of the effect of intracerebral BCG infection on neuronal phenotype and function in vivo. Neurons percentages were constant throughout infection. BCG infection at week 6

induced production of neuronal MHC class I but did not appear to have any significant effect on their capacity to produce cytokines (Figure 3-11)

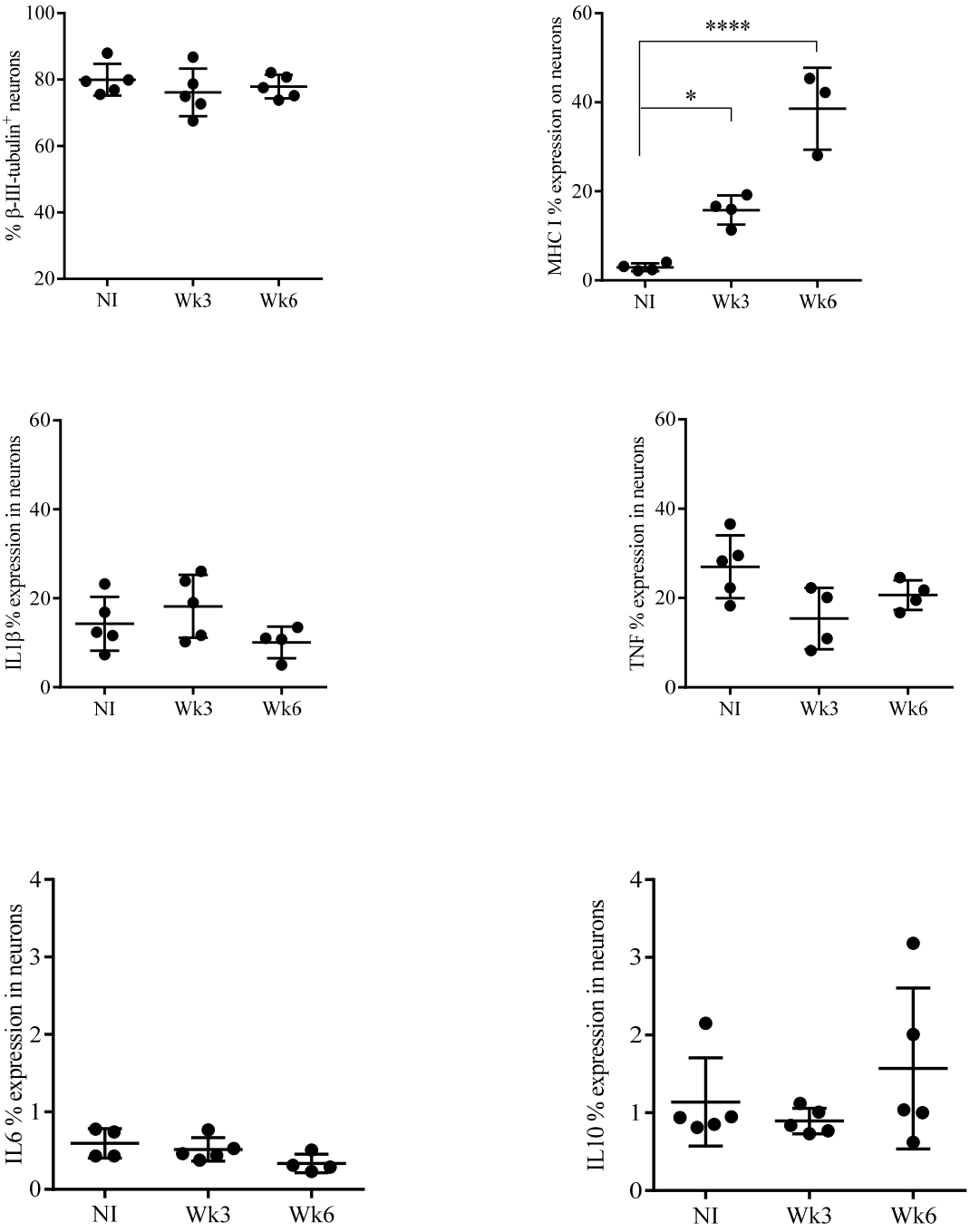


Figure 3-11: FACS analysis of neurons stained from whole brains of adult mice infected with BCG-GFP for 3-6 weeks. Results represent percentage of expression by β-III-tubulin^{+} neurons. 1x10^{6} cells were stained with respective antibodies prior to FACS analysis. NI – non-infected, Wk – week. Data are mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P <0.0001.

4. DISCUSSION

Numerous bacterial pathogens, such as *Mycobacterium tuberculosis* (Mtb) and *Listeria monocytogenes,* are able to invade the CNS to establish infection (Drevets et al., 2004). Several studies have reported on different types of CNS resident cells, such as neurons, microglia and astrocytes, targeted by Mtb for invasion. Due to the phagocytic nature of microglia, most of these studies focus on the relationship of Mtb bacilli with microglia (Shastri et al., 2013, Rock et al., 2005, Peterson et al., 1995). However, recent observations have shown that Mtb bacilli can be internalized by neurons and elicited neuronal responses (Randall et al., 2014, Francisco et al., 2015b). The data from this study are consistent with the previous findings and confirm the internalization of Mtb bacilli by neurons. Furthermore, neurons can also internalize BCG, a less pathogenic strain of Mtb. Interaction of neurons with live Mtb or BCG results in direct cell activation as reflected by changes in neuronal phenotype and cytokine expression in vitro and in vivo. Data also shows neurons are readily activated by BCG infection in vitro with minimal to no changes in vivo. Mycobacterium tuberculosis and BCG infection of neurons increased their expression of MHC class I but not MHC class II molecules, suggesting that infected neurons may have an increased ability to stimulate T cells (specifically $CD8^+$ T cells) against mycobacterial antigens as CD8 TCRs recognize antigenic peptides associated with MHC class I molecules. It has been long established that the interaction between $CD4^+$ T cells and macrophages are critical for immune protection against tuberculosis, particularly in bacterial clearance and containment (Orme et al., 1993, Ndlovu and Marakalala, 2016, Boom et al., 2003). Clinical importance of $CD4^+$ T cells have been demonstrated in HIV patients with increased risk for both tuberculosis infection and reactivation. Although initially $CD8^+$ T cells were believed to be

less important, increasing evidence has shown their essential role in this immune response to Mtb infection. In the absence of MHC class I presentation to $CD8^+$ T cells, the mice were susceptible to Mtb and BCG infection (Flynn et al., 1992, Ladel et al., 1995), Populations of $CD8^+$ T cells form part of T cells subsets mediating protection against respiratory mycobacterium challenge (Orme, 1987, Boom et al., 2003).

Mycobacterial infection of neurons induces the upregulation of inflammatory cytokines such as IL1β, IL6 and a non-significant expression TNF and regulatory cytokine IL10. Based on the percentage of neurons expressing of selected surface molecules and cytokines, Mtb infected neurons are more efficient than BCG infected neurons at eliciting specific cellular responses in vitro. This may be attributed to neuronal efficiency at internalizing virulent Mtb than avirulent BCG. A study demonstrated that human microglia internalize Mtb bacilli more than avirulent *Mycobacterium avium* resulting in lasting inhibition of IL1 and IL10 productions (Curto et al., 2004). However, this present study was not designed to show a clear association between strains of Mtb and frequency of neuronal responses. High expression of cytokines such as TNF-α have previously been shown to be related to extensive mycobacterial damage seen in animals infected with H37Rv strain (Nagesh Babu et al., 2008). The levels of cytokines produced may have a significant impact on neuronal disease and protective immunity against Mtb infections. Many studies have focused on pro- and anti-inflammatory cytokines responses to infection with pro-inflammatory cytokines considered traditionally harmful in the CNS. Initial inflammatory responses to pathogen neuronal invasion are not always bad as they provide signs that identify the CNS as a site of infection and results in the recruitment of leukocytes into infected CNS to promote pathogen containment and clearance. This is not to disregard the role of inflammatory responses in

worsening infection, resulting neuronal sequelae (Grimwood et al., 2000, Dawson et al., 1999). Therefore, immune response must maintain a careful balance between preventing apoptosis (due to non-regenerative nature of neurons) and containing infection. And regardless of the outcome to ensue following increased neuronal expression of inflammatory molecules, these data show neurons also play a role in immune reaction during Mtb and BCG challenge.

Animal models of cerebral tuberculosis have been established in mice (van Well et al., 2007, Zucchi et al., 2012, Wu et al., 2000). The experimental model was used in this study to reproduce more closely the human disease as in vitro system cannot mimic complex interactions of several cell types and compartments in vivo. Interestingly, the expression patterns of neuronal surface molecules (MHC class I) and cytokines (IL1β, IL6, TNF and IL10) at day 14 post intracerebral mycobacterial infection, corroborated with in vitro data. There was little to no neuronal response to BCG infection in vivo except for neuronal expression of MHC class I. This may be in line with cellular responses dependent on excessive inflammation associated with virulent pathogens. The levels of cytokines expression in vitro were low which may render them non-detectable in analysis of the whole brain where responses are affected the presence of other cell types.

The role of the immune system is to defend the body against infection and injury. CNS is no longer seen as an immune privileged site (Louveau et al., 2015). In CNS inflammatory conditions, T cells infiltrate the parenchyma and upon severe immune attack, damage takes place. T cells also play a role in tissue homeostasis and exert neuroprotective effects in neuronal injury (Walsh et al., 2015, Steinman, 2015, Gadani et al., 2015), autoimmunity (Kipnis et al., 2001, Kipnis et al., 2002) and neurodegenerative disorders (Stangel, 2012,

Maghzi et al., 2013). Therefore, this study sought to investigate functional responses of infected primary neurons after encounter with $CD3^+$ T cells. The consequences of such an encounter are important as they dictate the outcome of the disease. Current study demonstrated that neuronal contact with T cells leads to significant decrease in neuronal expression of MHC class I, IL1β and TNF by FACS analysis. And, ELISA analysis of supernatants has shown a decreased production of IL6 in neuron T cell co-cultures in comparison with the neuron only cultures. These data suggest possible cell death of infected neurons expressing MHC class I due to interaction with cytotoxic $CD8^+$ T cells. Neurons have been shown to express MHC class I in inflammatory setting (Foster et al., 2002, Chevalier et al., 2011, Neumann et al., 1997) and during neuronal development (Shatz, 2009, Corriveau et al., 1998, Chacon and Boulanger, 2013). Neuronal MCH class I expression in those settings was capable inducing $CD8^+$ T cells proliferation and triggering Fas (Fas on neurons and Fas ligand on $CD8^+$ T cells) (Meuth et al., 2009, Medana et al., 2000, Neumann et al., 1997) and perforin based cytotoxic neuronal killing (Cebrian et al., 2014). Another plausible scenario is neurons or Mtb/ BCG downregulates MHC class I to evade possible interaction with $CD8^+$ T cells. Reduction of cytokines such as IL1β, IL6 and TNF mediated by the presence of T cells may also be attributed to apoptosis of inflamed neurons induced by interaction with cytotoxic $CD8^+$ T cells. $CD4^+$ T cells cannot recognize antigens on neurons due to lack of MHC class II molecules but they have been shown to utilise alternative pathways to interact with neurons (Liu et al., 2006). IL10 production in supernatants of neuron T cell co-cultures indicates initiation of neuroprotective responses with the induction of Th2 regulatory T cells. IL10 expressed by brain Tregs have been shown to suppress the inflammatory responses of active microglia (Xie et al., 2015). These results indicate distinct

roles of $CD8^+$ and $CD4^+$ T cell subsets, supporting the concept of T cells have neuroprotective and/ or neurodegenerative roles. This is shown in previous co-culture experiments in which activated human $CD8^+$ T cells inhibit neurite outgrowth in a contact dependent manner whereas activated $CD4^+$ T cells promote neurite outgrowth (Pool et al., 2012, Walsh et al., 2015). In experimental models of CNS injury, T cells protect neurons and increase neuronal survival (Kipnis et al., 2002, Kipnis et al., 2001). Therefore, the understanding of neuroprotective and neurodegenerative contributions of T cells in mycobacterial infection of CNS warrants future studies.

Studies on immune functions of neurons have indicated that neurons participate in immune regulation of by controlling infiltrated T cells. It is well established that APCs are specialized for naïve T cell activation and normally APCs interact with T cells by priming and activating them. Molecules released in response to a pathological stimulus also influence the type of T cell responses. Neurons have been shown to have a direct immune regulatory role on T cells and CNS inflammation (Liu et al., 2006). Neurons have been shown to use direct and indirect communication with T cells to regulate T cell response. Present study investigated the immune regulatory roles of neurons under Mtb/ BCG inflammatory. Given the data obtained from the neuron T cell co-cultures of this study, it is very likely that neurons exposed to mycobacteria are able to successfully stimulate T cell responses and this is supported by increased expression of T cell transcription factors. Mtb infected neurons play a direct role in immune regulation during neuron T cell interaction via cell-to-cell contact and/ or via specific cytokine expression. Neurons were able to induce T cell transcription factors such as expression of Tbet for Th1, RorγT for Th17 and Gata3 for Th2 responses. Reponses seem to be dependent on direct contact with neurons in contrast to conditioned media and transwell

cultures which had no significant changes in both $CD4^+$ and $CD8^+$ populations and to a certain extent, independent of infection. ELISA analysis of co-culture supernatants showed an increased production of IFN-γ. It is plausible to suggest that recognition of specific MHC I peptide complexes by T cells promotes the release of IFN-γ which in turn enhances neuronal Fas expression and susceptibility to apoptosis. Enhanced Th1 cellular responses and increased production of IFN-γ are in line with reports in pulmonary TB where IFN-γ producing T cells are required for protective immunity and their arrival at the site of infection correlates with decreased bacterial growth (Green et al., 2013). Gata3 expression seen in $CD4^+$ population is also confirmed by IL10 production significantly increased in co-culture supernatants. It was confirmed in this study neurons do not express MHC class II, therefore $CD4^+$ T cells interaction with neurons will most likely be independent of MHCII/ TCR signalling T cell where responses do not necessarily require antigen recognition but rather induced by damage associated molecules expressed by neurons in response microbial antigens. These data confirm that neurons are capable of inducing T cell responses against mycobacteria.

Proposed future studies will include elucidating mechanism of bacilli uptake where scanning and transmission electron microscope could be utilised to visualise interaction of bacilli with cultured neurons. Though intracerebral Mtb infection has been widely used as a reproducible in vivo model of CNS TB (Hsu et al., 2017, Francisco et al., 2015a), it is still not the natural way CNS TB is acquired in humans. In the animal models, mice do not show clinical signs of the disease which are necessary for clinical diagnosis. Nonetheless, the model can provide localized Mtb infection and the subsequent immune responses in the brain, which is useful for further investigate neuron T cell interaction in vivo, the phenotype and effect of CNS

infiltrated T cells – as well as analyse variety of cytokines and receptors elucidating signalling pathways that orchestrate immunological responses to CNS TB. More experiments should have also been done to confirm progression of disease as CNS TB develops not directly by presence of bacilli in the meninges but by release of bacilli from focal lesions. Histological analysis of the whole brain would have highlighted the outcome of infection.

5. CONCLUSION

Traditionally, neurons are believed to be passive in the immune responses of the CNS. Considering the novel observations that neurons directly interact with Mycobacterium tuberculosis, neurons are capable of inducing immune responses and actively involved in CNS immunity. Recent findings have shown the immune regulatory role of neurons in many CNS disorders where they utilised varying mechanisms to influence T cell responses. This study suggests that neuronal responses to Mtb may preferentially drive T helper cells to mature into Th1 and/or Th2 cell types therefore controlling the generation of type 1 or type 2 immune responses to Mtb - allowing inflammatory response to clear pathogens as well as limit damage to the CNS. On the contrary, BCG infection affects neuronal phenotype and upregulates cytokine production but does not appear to participate actively in the activation and maturation of T cells. Applying immunological methodologies to neurological studies highlights interesting interactions between the immune and nervous system and shows that immune mechanisms are important in a wide range of neurological disorders. If neurons can induce an appropriate immune response to mycobacteria, then targeting mycobacterial antigens to neurons may form the basis of new generation of therapeutics against CNS-TB with improved outcomes in terms of immune protection and neurological morbidity. And add new understanding of the immune regulatory process during tuberculosis infection of the CNS with emphasis on the respective roles of neurons and immune cells.

APPENDIX A

1.1. **Table A-1**: FACS T Cell Antibody Panel

Antibody	**Fluorophore**	**Dilution**	**Clone No.**	**Company**
CD3	BV421	1:100	145-2C11	eBioscience
CD4	Alexa 700	1:100	RM4-5	BD Pharmingen
CD8	APC-CY7	1:100	53-6.7	BD Pharmingen
CD44	FITC	1:100	Pgp-1Ly-24	BD Pharmingen
T cell transcription factors				
Tbet	PE	1:50	O4-46	BD Pharmingen
RorYT	PERCP	1:50	Q31-378	BD Pharmingen
Gata3	PE-CY7	1:50	L50-823	BD Pharmingen
FoxP3	Alexa 647	1:50	MF23	BD Pharmingen

1.2. **Table A-2:** FACS Neuron Antibody Panel

Ab	**Fluorophore**	**Dilution**	**Clone No.**	**Company**
βTubulin	Alexa 647	1:50	EP1569Y	Abcam
MHC I	PE	1:100	AF6-88.55.3	eBioscience
MHC II	Alexa 700	1:50	M5/114.15.2	eBioscience
TNF	APC-CY7	1:50	MP6-XT22	BD Pharmingen
IL1β	PE-CY7	1:50	NJTEN3	eBioscience
IL10	BV711	1:50	JES5-16E3	BD Horizon

1.3. **Table A-3:** ELISA Antibodies and Standards

Antibody	**[Coating]**	**Company**	
IL1β	4µg/ml	R&D Systems	
IL4		R&D Systems	
IL6	2µg/ml	R&D Systems	
IL10	2µg/ml	R&D Systems	
TNFα	4µg/ml	R&D Systems	
Antibody	**[Detection]**	**Enzyme & Dilution**	**Company**
IL1β	400ng/ml	Strep AKP 1:1000	R&D Systems
IL4			R&D Systems
IL6	400ng/ml	Strep AKP 1:1000	R&D Systems
IL10	200ng/ml	Strep AKP 1:1000	R&D Systems
TNFα	200ng/ml	Strep AKP 1:1000	R&D Systems

Sample	[Recombinant]	Company
IL1β	2ng/ml	R&D Systems
IL4		R&D Systems
IL6	1ng/ml	R&D Systems
IL10	4ng/ml	R&D Systems
TNFα	1ng/ml	R&D Systems

Figure A-1: Gating strategy for splenic CD3+ T cells sort

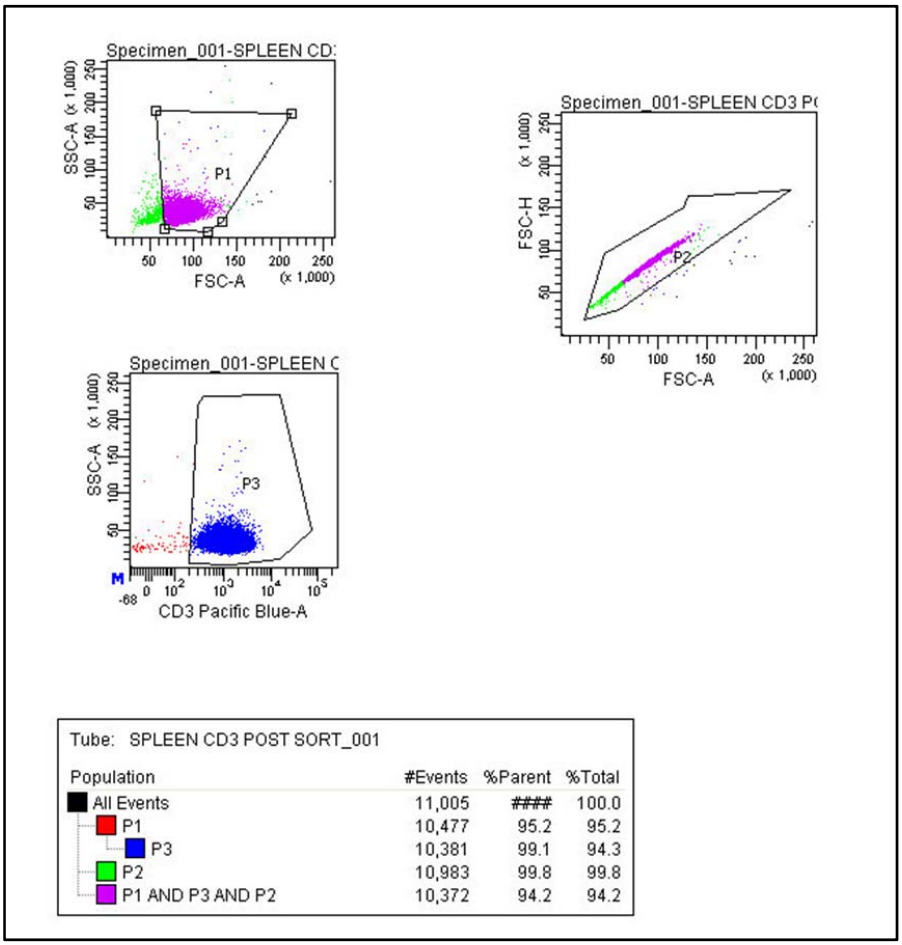


Figure A-2: Gating strategy for in vitro βtubulin^{+} neurons

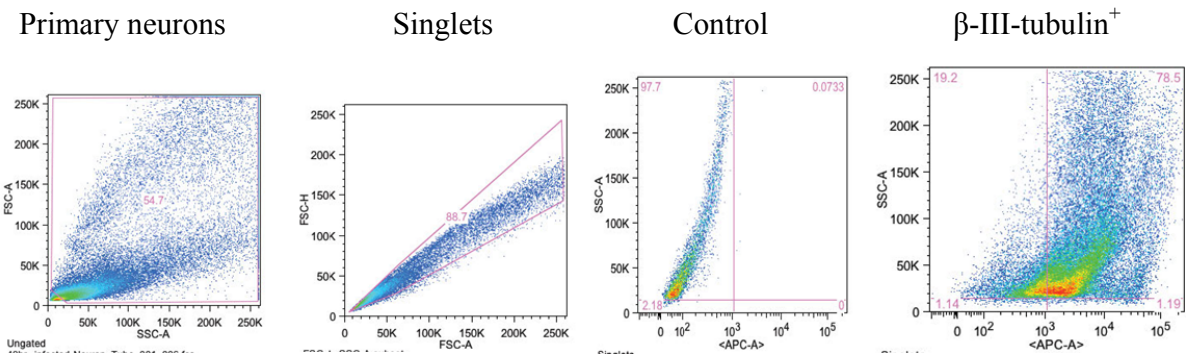


Figure A-3: Gating strategy for in vitro CD3+CD4+ and CD3+CD4+ T cells

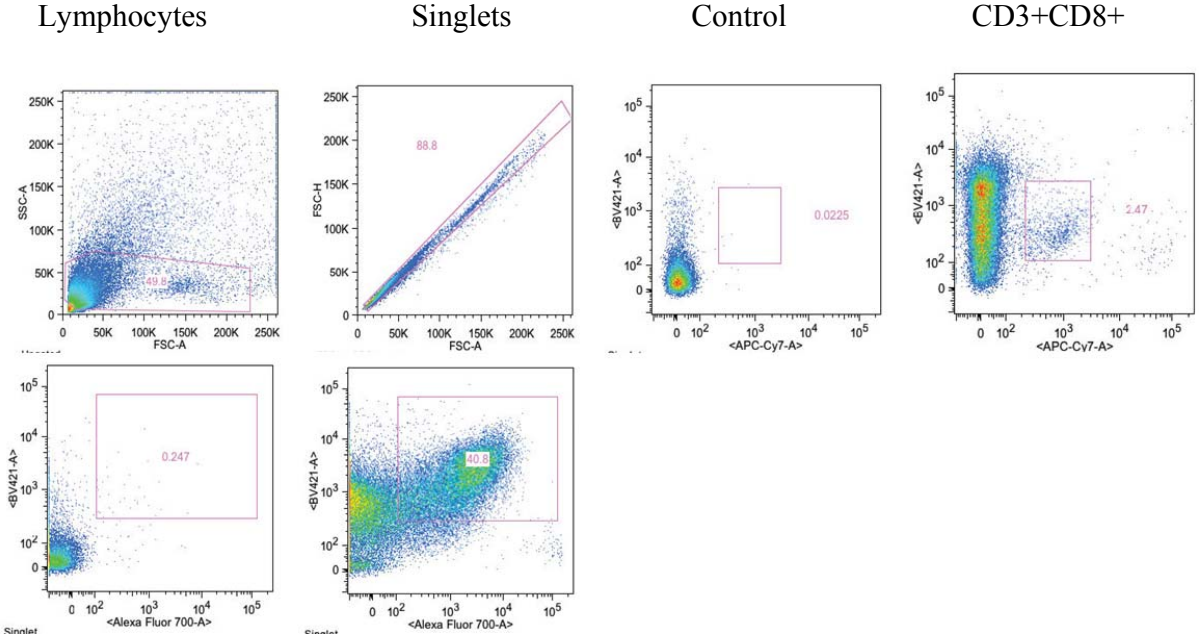
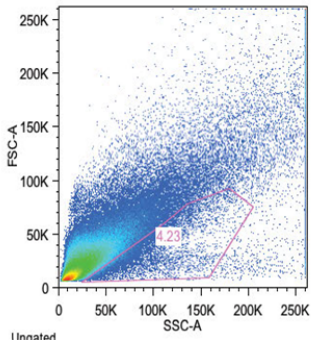
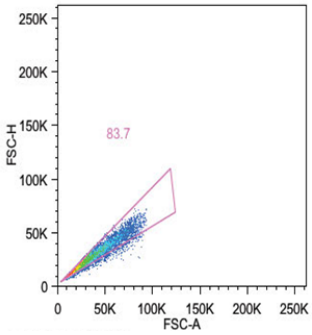


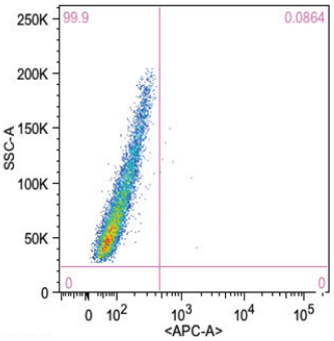
Figure A-4: In vivo βtubulin^{+} neuron gating

Neurons

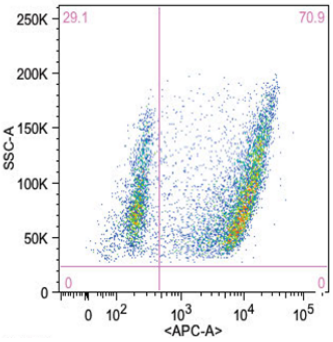


Singlets





Control



β-III-tubulin^{+}

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